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Abstract

Colorectal cancer (CRC) is one of the most common cancers and a leading cause of cancer-related mortality worldwide. Although systemic therapy is the standard care for patients with recurrent or metastatic CRC, the prognosis is extremely poor. The optimal sequence of therapy remains unknown. Therefore, alternative strategies, such as immunotherapy, are needed for patients with advanced CRC. This review summarizes evidence from dendritic cell-based cancer immunotherapy strategies that are currently in clinical trials. In addition, we discuss the possibility of antitumor immune responses through immunoinhibitory PD-1/PD-L1 pathway blockade in CRC patients.

Key words: Colorectal cancer; Dendritic cell; Cancer immunotherapy; Cytotoxic T lymphocyte; Immune-checkpoint inhibitors

Core tip: Dendritic cell (DC) is potent antigen-presenting cells that play a pivotal role in the induction of antitumor immune responses. Strategies for delivering antigens to DCs have been developed and used in clinical trials

in cancer patients, including colorectal cancer (CRC). Numerous reports indicate that the use of DC-based immunotherapy for CRC patients is promising to induce antigen-specific CTL responses. However, the immune suppression induced through CRC and the tumor microenvironment continues to be a major hurdle. Thus, the combination of DC-based immunotherapy with immune-modulating agents may be necessary to maximize antitumor immunity. These combinatorial therapies may have the potential for clinical benefit.

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INTRODUCTION

Colorectal cancer (CRC) is a common cancer and remains one of the leading causes of cancer-related deaths worldwide. Although surgery is the only curative treatment available for localized disease, more than 20% of CRC patients are not eligible for surgery due to liver metastases at the time of diagnosis[1]. To date, surgery, neoadjuvant radiotherapy and adjuvant chemotherapy have improved the outcome of CRC patients; however, 50% of patients still die from recurrent or metastatic disease[1,2]. Indeed, the treatment of CRC patients with distant metastases or recurrence through surgery or chemotherapy currently remains limited. Therefore, alternative strategies, including immunotherapy, for treating advanced CRC have been considered[3].

Recent studies have suggested that CRC is a good candidate for immunotherapy. As potential targets for cancer immunotherapy, human CRC cells express numerous numbers of tumor-associated antigens (TAAs), such as carcinoembryonic antigen (CEA)[4-6], Wilms' tumor gene 1 (WT1)[7,8], mucin 1 (MUC1)[4,9], melanoma-associated antigen gene (MAGE)[10-12], or p53[13]. Moreover, CRC is a heterogeneous disease with genetic and epigenetic characterizations, such as the mutation of oncogenes, microsatellite instability (MSI) phenotype, chromosomal instability (CIN) pathway, CpG island methylator phenotype (CIMP), and DNA hypomethylation[14]. For example, the MSI phenotype reflects various deficiencies in the DNA mismatch-repair system, leading to an increased mutation rate of oncogenes[15]. The CIN pathway in cancers reveals aneuploidy and chromosomal rearrangements[15]. Cancers with the CpG island methylator phenotype (CIMP) exhibit DNA methylation associated with the transcriptional inactivation of tumor-suppressor genes[15]. These genetic and epigenetic characterizations lead to multiple mutations of oncogenes, resulting in immunogenic CRC. Therefore, some patients with CRC may be effective candidates for immunotherapy. Moreover, immunotherapy mediates a potent antitumor effect when combined with chemotherapy and/or radiotherapy[16-18]. Indeed, cancer immunotherapy targeting these TAAs can be combined with surgery, radiotherapy, and conventional chemotherapy for treating patients with CRC. Interestingly, given the success of immune-checkpoint inhibitors in several tumors, we believe that cancer immunotherapy may also be combined with immune checkpoint blockade agents to induce efficient antitumor immunity in CRC patients.

ANTITUMOR IMMUNITY

T cells with the αβ T cell receptor (TCR) generally express $CD4^+$ or $CD8^+$ lineage markers and have primarily been classified as helper or cytotoxic subsets, respectively[19]. Major histocompatibility complex (MHC) class Ⅰ molecules on cancer cells bound to antigenic peptide derived from tumor-associated antigens (TAAs) are recognized by the TCR of $CD8^+$ T cells. However, $CD4^+$ T cells recognize peptides in association with MHC class Ⅱ molecules on antigen-presenting cells (APCs)[3,19]. The goal of cancer immunotherapy is to induce efficient antigen-specific cytotoxic $CD8^+$T cells (CTLs). The induction of efficient $CD8^+$ CTLs requires helper functions mediated through $CD4^+$ T cells *via* the production of cytokines, such as interleukin (IL)-2 and interferon (IFN)-γ, resulting in the maintenance of antigen-specific $CD8^+$ CTLs[20,21]. Therefore, the simultaneous interaction of the TCR of T cells with antigenic peptides/MHC class Ⅰ and class Ⅱ complexes on APCs is essential for the induction of $CD4^+$ and $CD8^+$ T cell-mediated antitumor immune responses. Moreover, antigen-specific $CD8^+$ CTLs respond to antigenic peptides presented by MHC class Ⅰ molecules on cancer cells and identify and kill TAA-expressing cancer cells.

Dendritic cells (DCs) are potent APCs that play a pivotal role in the initiation, programming, and regulation of antitumor immune responses[20]. DCs capture antigens, resulting in a mature phenotype and the release of IL-12 from DCs. The exogenous antigens are processed by DCs, and antigenic peptides are presented on MHC class Ⅰ molecules, a process known as antigen cross-presentation[20]. In addition, DCs also process endogenously synthesized antigens into antigenic peptides, presented to MHC class Ⅰ molecules. However, exogenous antigens are also processed to antigenic peptides and complexed with MHC class Ⅱ molecules[20,21]. Antigen presentation primarily occurs in the draining lymph node, where antigenic peptides are presented by DCs, resulting in the simultaneous activation of $CD4^+$ and $CD8^+$ T cells. Moreover, interactions between DCs and innate and innate-like immune cells, such as natural killer (NK), invariant natural killer T (iNKT), and γδ T cells,

can bypass the T helper arm in CTL induction[22,23]. NK, iNKT, and γδ T cells also have the ability to attack tumor cells directly[23]. Therefore, efficient induction of antitumor immunity *via* DC-based cancer vaccines may require interaction between DCs and innate and innate-like immune cells with central roles in DC-based cancer immunotherapy[23,24].

Cancer immunotherapy, including peptide vaccines, whole tumor cell vaccines, viral vector vaccines, and adopted cell transfer therapy, have been developed to treat CRC patients[3]. In particular, peptide vaccines have been widely tested in clinical trials, reflecting the simple, safe, stable, and economical features of these vaccine types. However, there are several drawbacks to the peptide vaccines, including: (1) limitations due to the MHC type; (2) limited numbers of identified epitopes; and (3) impaired DC function in cancer patients[3,25]. Therefore, DCs have been loaded with multiple antigenic peptides[26-28], whole tumor cell-mRNA[29], whole tumor cell lysates[30], and whole tumor-derived apoptotic bodies[31] or fused with whole tumor cells to form hybrid cells (DCs-tumor fusions)[32]. DC-tumor fusion cells process a broad array of TAAs, including both known and unidentified, and present these molecules by MHC class Ⅰ and class Ⅱ pathways in the context of co-stimulatory molecules[32,33]. In our laboratory, patient-derived DCs are generated through adherent mononuclear cells from a single leukapheresis collection after culture in the presence of granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-4. Immature DCs are matured with penicillin-killed and lyophilized preparations of a low-virulence strain (Su) of *Streptococcus pyogenes* (OK-432) and with prostaglandin E2 (PGE2). Subsequently, a large number of DCs can be cryopreserved in ready-for-use aliquots for immunotherapy[27].

IMMUNOSUPPRESSION MECHANISMS

Although antigen-specific CTLs are induced in cancer patients, cancer cells often escape immune surveillance through several mechanisms, including (1) the down-regulation of certain antigens, TAP-1/2, MHC class Ⅰ, or peptide-processing machinery in tumor cells[34,35]; (2) the induction of regulatory T cells (Tregs) producing proinflammatory and immunosuppressive cytokines, such as IL-10 and TGF-β[36]; (3) the presence of immunosuppressive cells (*e.g.,* cancer-associated fibroblasts (CAFs), M2 macrophages, myeloid-derived suppressor cells (MDSCs), immunosuppressive tumor-associated macrophages (TAMs), tolerogenic DCs, and Tregs) in the tumor microenvironment[36]; (4) the production of multiple immune suppressive factors from tumor cells[37]; and (5) the expression of immune checkpoint blockade between tumor cells and activated T cells[38,39]. Although, activated $CD8^+$ T cells associated with clinical prognosis often infiltrate in CRC[40], this benefit is controlled through immune suppressive cell populations in the tumor microenvironment, promoting tumor escape from immune surveillance[14,41,42]. The direct production of immune suppressive factors, such as IL-6, IL-10, TGF-β, vascular endothelial growth factor (VEGF), soluble Fas ligand (Fas-L), and indolamine-2,3-dioxygenase (IDO), by tumor cells also promotes the accumulation of heterogeneous populations of CAFs, M2 macrophages, TAMs, MDSCs, tolerogenic DCs, and Tregs[37]. These immunosuppressive cells in the tumor microenvironment inhibit antitumor immunity through various mechanisms, including the elaboration of arginase (Arg), nitrogen oxide (NO), and reactive oxygen species (ROS) from immunosuppressive cells[37]. Indeed, the tumor microenvironment is extremely complex and suppresses antitumor immunity, thus explaining why cancer immunotherapy is occasionally unsuccessful[41]. Therefore, the functional inhibition of Arg, NO, or ROS in immunosuppressive cells may augment antitumor immunity.

Programmed death 1 (PD-1) is expressed on the surface of activated T cells and inhibits T cell activation upon binding to the associated ligands PD-L1/PD-L2[3]. Moreover, cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is another mechanism that inhibits T cell activation upon binding CD80/CD86 on DCs. CTLA-4 is expressed on naive or memory T cells. PD-1 is highly expressed in antigen-specific CTLs and activated DCs. PD-L1 is not constitutively expressed in some tumors but is induced in response to inflammatory signals, such as IFN-γ, produced by antigen-specific CTLs. In contrast, the CTLA-4-mediated immune checkpoint is induced in T cells during the initial response to antigen. Therefore, antibodies can be used to block inhibitory ligand-receptor interactions by acting on tumor cells and DCs (*e.g.*, anti-PD-L1) or T cells (*e.g.*, anti-CTLA-4 or anti-PD1). Indeed, CRC cells express PD-L1 associated with CTL inactivation and Treg development in the tumor microenvironment, resulting in worse survival[43-45]. Combining the blockade of multiple immune inhibitory pathways may synergistically activate antitumor immunity.

DC-BASED PASSIVE IMMUNOTHERAPY

DC-based cancer immunotherapy has been developed to induce TAA (*e.g.,* CEA, WT1, MAGE, or MUC1)-specific CTLs in patients with CRC. To date, various strategies for delivering TAAs to DCs have been developed and tested in clinical trials in cancer patients, including CRC (Table 1). In particular, as most CRC cells express CEA, CEA-targeted DC-based CRC immunotherapy has been reported.

CEA

CEA is a so-called onco-fetal antigen abundantly present in a majority of CRC cases. Importantly, the elevated expression of CEA is associated with adenocarcinoma, particularly CRC. Therefore, CEA-

Table 1 Clinical trials of dendritic cell-based cancer immunotherapy in patients with colorectal cancer

Targets	Immunotherapy	Phase	Patients	Results	Ref.	Year
CEA	DCs loaded with CEA peptide (HLA-A2 restricted)	I	21 advanced CEA-expressing malignancies including 11 CRC	Skin punch biopsy at DC injection sites demonstrated pleomorphic, perivascular infiltration of cells consistent with a DTH response	Morse *et al*[46]	1999
	DCs loaded with CEA mRNA	I	13 patients with resected hepatic metastases of CRC	9 of the 13 CRC patients relapsed at a median of 122 d	Morse *et al*[47]	2003
	DCs modified with a recombinant fowlpox vector encoding CEA and a triad of costimulatory molecules [rF-CEA(6D)-TRICOM]	I	14 patients with HLA-A2 (11 CRC and 3 non-small cell lung cancer)	CEA-specific T cells responses were detected in 10 patients; 5 patients were stable through at least 1 cycle of immunization (3 mo)	Morse *et al*[48]	2005
	Fowlpox vector encoding CEA	I	14 patients (5 CRC, 3 lung cancer, and 1 urachal adenocarcinoma)	Of the 9 patients analyzed, all with stable disease (n = 5) displayed increased NK activity	Osada *et al*[49]	2006
	Mature DCs induced by activation with a combination of OK-432, low-dose prostanoid, and IFN-α and loaded with CEA peptide		10 CRC patients	CRC patients with stable disease (n = 8) exhibited increased levels of NK cell frequency and CEA-specific CTL activity with a central memory phenotype. Lack of CTL activity was found in 2 CRC patients with progressive disease, but NK cell proliferation was detected	Sakakibara *et al*[51]	2011
	DCs loaded with altered CEA peptide (HLA-A2 restricted) with Flt3 ligand	I	12 patients with HLA-A2$^+$ malignancies (10 CRC and 2 non-small cell lung cancer)	CEA-specific CD8$^+$ CTLs were detected in 7 patients; 1 patient with progressive metastatic CRC had a complete resolution of pulmonary metastasis and malignant pleural effusion at 4 mo after vaccination, and 1 patient with CRC developed a mixed response after vaccination, with regression of some but not all liver metastases	Fong *et al*[52]	2001
	DCs loaded with CEA peptide (HLA-A24 restricted)	I	10 advanced CEA-expressing malignancies including 7 CRC	2 patients (CRC and lung cancer) exhibited positive DTH reactions against CEA and remained stable for 6 and 9 mo, respectively	Itoh *et al*[53]	2002
	DCs loaded with CEA peptides (HLA-A2- or HLA-A24-restricted)	I	10 CRC patients (6 HLA-A24 and 4 HLA-A2) who had failed standard chemotherapy	CEA-specific CTLs were detected in 7 patients; 2 patients exhibited stable disease for at least 12 wk	Liu *et al*[54]	2004
	DCs loading with CEA peptide (HLA-A24 restricted)	I	8 patients with advanced CEA-expressing gastrointestinal malignancies (7 CRC and 1 gall bladder cancer)	4 patients developed CEA-specific CTL responses; a DTH reaction was observed in 1 patient, with skin biopsy at the injection site showing lymphocyte infiltration, and 3 patients, including 2 CRC, exhibited stable disease after vaccination	Matsuda *et al*[55]	2004
	DCs loaded with CEA peptide (HLA-24 restricted)	I	8 patients with CEA-expressing metastatic gastrointestinal or lung adenocarcinoma	Long-term stable disease or marked decreases in the serum CEA level was observed in some patients. CEA-specific immune responses were demonstrated in most of the patients in whom treatment was clinically effective	Ueda *et al*[56]	2004
	DCs loaded with CEA peptide (HLA-2 restricted)	I	10 CRC patients with resection of liver metastases	CEA-specific CTLs were demonstrated in 7 patients; CEA-specific CTLs were detected in a resected lymph node in 1 patient	Lesterhuis *et al*[57]	2006
	DCs loaded with CEA altered peptide	I	9 patients with CEA-expressing malignancies (7 CRC and 2 lung cancer)	5 patients exhibited CEA altered peptide-specific CTL responses, and 3 patients exhibited CEA-specific CTL responses	Babatz *et al*[58]	2006
WT1	DCs loaded with WT1 peptide (MHC class Ⅰ and class Ⅱ restricted)	I	3 advanced CRC	WT1-specific CTLs were detected and persisted for 2 yr with prolonged disease-free and overall survival	Shimodaira *et al*[8]	2015

MAGE	DCs loaded with MAGE-3 peptide (HLA-A2 or A24 restricted)	I	12 patients with advanced gastrointestinal carcinoma (6 stomach, 3 esophagus, and 3 CRC)	MAGE-3-specific CTL responses were observed in 4 patients. Tumor markers were decreased in 7 patients, and evidence of minor tumor regression was detected in 3 patients	Sadanaga *et al*[65]	2001
	DCs loaded with MAGE-3 or MAGE-1 peptides (HLA-A2 0r A24 restricted)	I	28 patients with advanced gastrointestinal carcinoma, including 7 CRC	Peptide-specific CTL responses, tumor marker decreases, and minor tumor regressions were observed in some patients after vaccination	Tanaka *et al*[66]	2008
CEA and MUC1	DCs modified with CEA/MUC1 (PANVAC)	II	74 patients, disease free after CRC metastasectomy and perioperative chemotherapy	CEA-specific CTLs were detected	Morse *et al*[67]	2013
CEA, MAGE, and HER2	DCs loaded with CEA/MAGE/HER2/neu/pan-DR peptides (HLA-A2 restricted) and keyhole limpet hemocyanin (KLH) protein	I	13 advanced CRC	All patients exhibited progressive disease. CEA-specific CTLs were detected in 3 of 11 evaluated patients. Multiple TAAs-specific CTLs were induced	Kavanagh *et al*[70]	2007
Autologous whole tumor mRNA	DCs transfected with whole-tumor mRNA	I	15 advanced CRC received the immunotherapy and KLH intravenously	11 of the 13 CRC patients evaluated developed a positive KLH skin test, and 7 CRC patients exhibited CEA-specific responses	Rains *et al*[75]	2001
Autologous whole tumor cells	DCs-autologous whole-tumor fusion cells and IL-12	I	5 gastrointestinal tumors, including CRC	Among the 3 patients evaluated, 1 exhibited stable disease, and 2 exhibited progressive disease. No DTH-positive patients were detected in this trial. Good therapeutic responses in some patients with brain tumors were detected	Homma *et al*[78]	2005
Allogeneic whole tumor cell lysate	DCs loaded with allogeneic tumor cell lysate	I	6 advanced CRC (HLA-A2)	Antitumor immune responses in some patients and transient stabilization or even reduction of CEA levels were detected	Tamir *et al*[82]	2007
	DCs loaded with allogeneic melanoma cell lysate expressing at least one of six MAGE-A antigens	II	20 advanced CRC	1 patient experienced a partial response, 7 patients achieved stable disease, and 5 patients exhibited prolonged progression-free survival	Toh *et al*[83]	2009

DC: Dendritic cell; CRC: Colorectal cancer; DTH: Delayed-type hypersensitivity; CTL: Cytotoxic T lymphocyte.

targeted cancer immunotherapy has been developed. Morse *et al*[46] first conducted a phase I study using DCs loaded with an HLA-A2-restricted CEA peptide for the treatment of patients with 21 advanced CEA-expressing malignancies, including 11 CRC cases. One patient with ovarian cancer had a minor response, and one patient with breast cancer exhibited stable disease. Skin punch biopsy at DC injection sites demonstrated the pleomorphic, perivascular infiltration of cells consistent with a delayed-type hypersensitivity (DTH) response. This group also reported a phase II study of 13 patients with resected hepatic metastases of CRC, who received DCs loaded with CEA mRNA (DC/CEA mRNA). The administration of DC/CEA mRNA to CRC patients was feasible and safe. Nine of the 13 patients relapsed at a median of 122 days[47]. Furthermore, DCs modified with a recombinant fowlpox vector encoding CEA and a triad of costimulatory molecules [rF-CEA(6D)-TRICOM] was developed from the same group[48]. In this trial, 14 patients with HLA-A2 (11 with CRC and 3 with non-small cell lung cancer) were enrolled. CEA-specific T cells responses were detected in 10 patients. Five patients were stable through at least one cycle of immunization (3 mo)[48]. As recent reports indicate that DC-NK cell interaction plays a critical role in the induction of antitumor immunity[23,24], the same group conducted a phase I clinical trial of a vaccine consisting of autologous DCs loaded with a fowlpox vector encoding CEA[49]. Fourteen patients (5 CRC, 3 lung cancer, and 1 urachal adenocarcinoma) were enrolled in the trial; of the 9 patients analyzed, all with stable disease (n = 5) exhibited increased NK activity. Therefore, NK responses following DC vaccination may correlate with clinical benefit, and evaluation of NK responses should accordingly be included as a biomarker for DC-based cancer vaccines in clinical trials[49,50]. Another recent clinical trial also supports the importance of NK activity in CEA peptide-loaded DC-based cancer vaccines. In this trial, mature DCs activated by a combination of OK-432, low-dose prostanoid, and IFN-α were used[51], loaded with the CEA peptide and administrated to 10 CRC patients. Interestingly, the CRC patients with stable disease (n = 8) exhibited increased levels of NK cell frequency and CEA-specific CTL activity with a central memory phenotype. Conversely, a lack of CTL activity was observed in those with progressive disease, even though NK cell proliferation was detected. To induce efficient CEA-specific CTL responses, another study developed altered CEA peptides restricted with HLA-

A2-loaded DCs, which were administered along with Flt3 ligand, a hematopoietic growth factor, to 12 patients with CRC (n = 10) or non-small cell lung cancer (n = 2)[52]. After vaccination, the expansion of CEA-specific $CD8^+$ CTLs was detected in 7 out of 12 patients. Interestingly, 2 out of 12 CRC patients experienced dramatic tumor regression. One patient with progressive metastatic CRC had a complete resolution of pulmonary metastasis and malignant pleural effusion at 4 mo after vaccination, and one patient with CRC developed a mixed response after vaccination, with the regression of some but not all liver metastases. Clinical trials of DCs loaded with HLA-A24 restricted CEA peptides have also been reported. The vaccines were injected with adjuvant cytokines, such as natural human interferon alpha (IFN-α) and natural human tumor necrosis factor alpha (TNF-α), in patients with 10 advanced CEA-expressing metastatic malignancies, including 7 CRC cases[53]. Two patients (CRC and lung cancer) exhibited positive DTH reactions against CEA remained stable for 6 mo and 9 mo, respectively. Therefore, HLA-A24 and A2-restricted CEA peptide might be useful for inducing CEA-specific immune responses. Liu *et al*[54] immunized 10 metastatic CRC patients (6 patients with HLA-A24 and 4 with HLA-A2) who failed standard chemotherapy with DCs loaded with HLA-A2- or HLA-A24-restricted CEA peptides. In this clinical trial, the DC vaccine was injected into one inguinal lymph node under sonographic guidance. After vaccination, CEA-specific T cells were detected in 7 out of 10 patients. Two patients exhibited stable disease for at least 12 wk. Matsuda *et al*[55] also conducted a pilot study of DCs loaded with HLA-A24-restricted CEA peptide for 8 patients with advanced CEA-expressing gastrointestinal malignancies (7 CRCs and 1 gall bladder cancer). Four out of 7 patients developed CEA-specific CTL responses after vaccination. A DTH reaction was observed in 1 patient. Skin biopsy at the injected site showed the infiltration of lymphocytes. Three patients, including 2 CRCs, exhibited stable disease after vaccination. Reports from clinical trials using DCs loaded with HLA-restricted CEA peptide vaccines have also been reported in Japan, as 60% of the Japanese population and some Caucasians express HLA-A24. Ueda *et al*[56] injected the vaccines into 8 patients with CEA-expressing metastatic gastrointestinal or lung adenocarcinomas positive for HLA-A24. In this trial, no definite tumor shrinkage was observed; however, long-term stable disease or marked decreases in the serum CEA level was observed in some patients after therapy. CEA-specific immune responses have also been demonstrated in most of the patients in whom treatment was clinically effective. Another study examining the vaccination of patients with resectable liver metastases from CRC using mature DCs loaded with HLA-A2-restricted CEA-peptide has been reported in the Netherlands[57]. A total of 10 CRC patients with resection of liver metastases were treated, and the induction of CEA-specific T cells was demonstrated in 7 out of 10 patients. Interestingly, CEA-specific CTL responses were detected in a resected lymph node in one patient. CEA altered peptide (CEAalt) was also administered with DCs to induce antitumor immunity in patients with CEA-positive CRC (n = 7) or lung cancer (n = 2)[58]. In this trial, 5 out of 9 patients exhibited CEAalt-specific CTL responses, and 3 of 9 patients exhibited CEA-specific CTL responses[58]. As CEA is typically produced in gastrointestinal tissue during fetal development, the immune system exhibits some degree of tolerance. Therefore, a break in tolerance is required to induce efficient CEA-specific immunity.

WT1

The *WT1* gene possesses oncogenic functions and is highly expressed in various types of malignancies, including CRC[59]. Moreover, WT1 expression in CRC is significantly associated with tumor progression, lymph node metastasis, distant metastasis and clinical stage[60]. Therefore, the WT1 protein may be one of the most promising cancer antigens. Indeed, the National Cancer Institute (NCI) has ranked WT1 as the number 1 target for cancer immunotherapy based on several factors[61]. Moreover, *WT1* expression may be essential for maintaining the transformed characteristics of cancer cells. Tumor escape from immune surveillance, reflecting the downmodulation of WT1, is unlikely to occur[62,63]. Therefore, WT1-specific immune responses for the elimination of tumors may be induced in many types of cancers. Shimodaira *et al*[8] conducted a phase Ⅰ study to investigate the safety and immunogenicity of DCs loaded with WT1 peptides restricted by MHC class Ⅰ and class Ⅱ (DC/WT1-I/Ⅱ) for advanced CRC patients. Standard treatment comprising surgical resection and chemotherapy was followed by 1 course of 7 biweekly administrations of DC/WT1-I/Ⅱ with OK-432 in 3 CRC patients. Importantly, WT1-specific CTLs were detected after the first vaccination and persisted for two years with prolonged disease-free and overall survival (OS)[8]. The maintenance of long-term WT1-specific memory $CD8^+$ T cells through DC/WT1-I/Ⅱ may be associated with clinical benefits in cancer patients[64].

MAGE

MAGE is a cancer-testis antigen aberrantly expressed in various types of human malignancies, including CRC. MAGE is not expressed in normal tissues except the testis. Thus, MAGE has been developed as a cancer immunotherapy target[10-12]. Sadanaga *et al*[65] initially examined DCs loaded with MAGE-3 peptide in patients with gastrointestinal carcinomas, depending on the HLA haplotype (HLA-A2 or A24). Twelve patients with advanced gastrointestinal carcinoma (six stomach, three esophagus, and three colon) were enrolled. After vaccination, MAGE-3-specific CTL responses were

observed in 4 out of 8 patients. Tumor markers were decreased in 7 patients, and importantly, evidence of minor tumor regression was detected in 3 patients. This group also conducted clinical trials for CRC patients using MAGE-3 or MAGE-1 peptide[66]. Twenty-eight patients with advanced gastrointestinal carcinoma, including 7 CRCs, were administered mature DCs loaded with MAGE-3 or MAGE-1 peptide, depending on the HLA haplotype (HLA-A2 or A24). Peptide-specific CTL responses, tumor marker decreases and minor tumor regressions were observed in some patients after vaccination.

CEA and MUC1

A recent report from a randomized phase Ⅱ clinical trial also indicated the clinical benefits of TAA-targeted DC-based cancer immunotherapy for CRC patients[67]. The aim of this trial was to determine whether 1 of 2 vaccines based on DCs and poxvectors encoding CEA and MUC1 (PANVAC)[68] would lengthen the survival of patients with resected CRC metastases. A total 74 patients, disease-free after CRC metastasectomy and perioperative chemotherapy, were randomized to injections of DCs modified with MUC1 PANVAC (DC/PANVAC) or PANVAC with per injection GM-CSF. The results indicated no differences in the clinical outcomes [progression-free survival (PFS) or OS] between the 2 vaccine strategies. Although CEA-specific T cell responders after DC/PANVAC were more frequently detected compared with PANC, the clinical benefits were not significant[67].

CEA, MAGE, and HER2

HER2/neu is a proto-oncogene product overexpressed in CRC cells[69]. Therefore, Kavanagh *et al*[70] conducted a phase Ⅰ/Ⅱ clinical trial administering a DC-based cancer immunotherapy targeting multiple TAAs, including CEA, MAGE, and HER2/neu, to patients with advanced CRC. The DCs were loaded with HLA-A2-restricted peptides derived from CEA, MAGE, and HER2/neu, pan-DR non-natural peptide optimized for both HLA-DR binding and TCR stimulation, and keyhole limpet hemocyanin (KLH) protein[71]. In this trial, 13 HLA-A2$^+$ advanced CRC patients received the immunotherapy. Although, all patients exhibited progressive disease, CEA-specific T cell responses were detected in 3 out of 11 evaluated patients. Moreover, this pilot study demonstrated the induction of immune responses to multiple TAAs in patients with advanced CRC.

DCs loaded with whole tumor cell-derived antigens

DCs can present TAA-derived epitopes in various manners. Unlike antigenic peptide-loaded DCs, other strategies, such as DCs loaded with whole tumor cells (DC/whole tumor) through whole tumor lysates, apoptotic whole tumor cells, DNA, mRNA, or fusion with whole tumor cells, have been developed[72]. DC/whole tumor cells simultaneously induce numerous TAA-specific CD4$^+$ and CD8$^+$ T cell responses that are at least theoretically more effective than antigenic peptide-loaded DCs[72]. Moreover, for DC/whole tumor-based immunotherapy, allogeneic tumor cell lines can also be used instead of autologous tumor cells to induce autologous tumor specific antitumor immunity. However, unlike defined antigenic peptides, whole tumor cell-based therapy is applicable to all patients, regardless of HLA type.

DCs transfected with mRNA

We have previously reported that murine DCs transfected with MUC1 mRNA exhibited MUC1 expression on DCs in the context of co-stimulatory molecules, resulting in the induction of MUC1-specific CTL responses against CRC cells *in vivo* and *in vitro*[73]. Comparative studies have suggested that mRNA-transfected DCs are superior to other antigen-loaded DCs in inducing CTL responses[29,74]. In a clinical trial, DCs were transfected with whole tumor mRNA to induce antitumor immunity in CRC patients[74]. Fifteen patients with advanced CRC received the immunotherapy and KLH intravenously[75]. As a result, 11 out of the 13 CRC patients evaluated developed a positive KLH skin test, and 7 CRC patients exhibited CEA-specific responses.

Fusion of DCs with whole tumor cells

The fusion of DCs with whole tumor cells generates a heterokaryon expressing DC-derived co-stimulatory molecules and a broad array of TAAs, including both known and unidentified molecules. Thus, this method offers several advantages for presenting antigenic peptides and subsequently inducing polyclonal antigen-specific CD4$^+$ cells and CD8$^+$ T cell-mediated antitumor immune responses, resulting in long-term antitumor immunity activation without inducing tolerance[76]. Moreover, this strategy circumvents the daunting task of identifying TAAs for individualized immunotherapy. Interestingly, DC-tumor fusion cells are potent immune stimulators compared with DCs loaded with either apoptotic tumor-cell fragments or tumor lysates in mice studies[77]. In DC-tumor fusion cells, TAAs access the endogenous antigen-processing pathway, whereas DCs loaded with apoptotic tumor-cell fragments or tumor lysates rely on the cross-presentation of the antigen, which is typically not efficient[33]. In a phase Ⅰ study, DC-tumor fusion cell vaccines were also administered with IL-12 in 5 gastrointestinal tumors, including CRC[78]. Among the 3 patients evaluated, 1 patient exhibited stable disease, and 2 patients exhibited progressive disease. Moreover, no DTH-positive patients were detected in this trial. Immunotherapy through DC-tumor fusion cells with IL-12 induced no serious adverse events and provided good therapeutic responses in some patients with brain tumors. In addition, patients with elevated serum levels of anti-nuclear antibody (ANA) had significantly longer treatment periods than those without treatment in these trials[79].

Table 2 Clinical trials of immune checkpoint therapy in patients with colorectal cancer

Target	Immunotherapy	Phase	Patients	Results	Ref	Year
PD-1	Pembrolizumab, anti-PD-1 immune checkpoint inhibitor	Ⅱ	11 MMR-deficient CRC, 21 MMR-proficient CRC, and 9 MMR-deficient non-CRC	The immune-related objective response rate and immune-related progression-free survival rate were 40% (4 of 10 patients) and 78% (7 of 9 patients), respectively, for MMR-deficient CRC and 0% (0 of 18 patients) and 11% (2 of 18 patients) for MMR-proficient CRC	Le *et al*[89]	2015

PD-1: Programmed death 1; CRC: Colorectal cancer; MMR: DNA mismatch repair.

Allogeneic whole tumor cell lysate-loaded DCs

The use of autologous whole tumor cell lysates as a potential source of TAAs for DC loading has several potential advantages compared with defined antigenic peptides. DC-loaded whole tumor cell lysates and DC-tumor fusion cells express both known and unidentified TAAs, circumventing the daunting task of identifying TAAs. Moreover, DC-loaded whole tumor cell lysates also induce the simultaneous activation of polyclonal $CD8^+$ and $CD4^+$ T cells[80,81]. The activation of $CD4^+$ and $CD8^+$ T cells can provide robust assistance for the induction and maintenance of $CD8^+$ CTLs. However, autologous whole tumor cell-based immunotherapy is often limited by the availability of sufficient numbers of autologous tumor cells, which may not be obtained when surgery is not a component of the treatment. Therefore, an alternative approach involves a use of allogeneic tumor cell lines instead of autologous tumor cells. This approach is based on the fact that some TAAs, such as CEA, WT1 and MUC1, are shared among most tumors. We demonstrated that allogeneic CRC cell-loaded autologous DCs induce antigen-specific CTL responses in CRC patients *in vitro*[4].

In clinical settings, autologous tumor lysate-loaded DC vaccines were used in advanced patients with CEA-positive CRC cells[82]. Six HLA-$A2^+$ CRC patients received the immunotherapy and tetanus toxoid antigen, hepatitis B, and influenza matrix peptides. The results revealed antitumor immune responses in some patients, and the transient stabilization or even reduction of CEA levels were also detected. Moreover, DCs loaded with allogeneic melanoma cell lysate expressing at least one of six MAGE-A antigens were examined in this phase Ⅱ study[83]. Twenty patients with advanced CRC received a total of 161 vaccinations. One patient experienced a partial response. Seven patients achieved stable disease. Five patients exhibited prolonged PFS.

IMMUNE CHECKPOINT THERAPY

DNA mismatch repair (MMR) is a group of genes encoding four proteins that play a key role in repairing mistakes and maintaining genomic stability[84]. Deficiencies in MM lead to MSI; thus, CRCs with MSI contain 10- to 100-fold more somatic mutations than metastatic CRC without MSI[85-87]. MSI reflects defective MMR in 15% to 20% of CRC patients[88]. Accumulating evidence indicates that the neoantigens produced from mutated proteins in tumors with MSI are recognized by the immune system, inducing CTL infiltration in tumors[87]. In addition, $CD8^+$ CTL infiltration in CRC has a well-supported prognostic value[42]. However, the tumor microenvironment comprises not only $CD8^+$ CTLs but also immune regulatory cell populations. Recent evidence indicates that $CD8^+$ CRLs infiltration in tumors is associated with the therapeutic effects of immune checkpoint strategies[42]. Le *et al*[89] conducted a phase Ⅱ trial to evaluate the clinical benefit of an anti-PD-1 immune checkpoint inhibitor, pembrolizumab, in 41 patients (11 MMR-deficient CRC, 21 MMR-proficient CRC, and 9 MMR-deficient non-CRC) (Table 2). Most patients (40 of 41) had previously received treatment of two or more lines of therapy; all patients then received pembrolizumab until either disease progression or unacceptable toxicity occurred. Pembrolizumab was well tolerated, and the immune-related objective response rates for MMR-deficient CRC and MMR-proficient CRC were 40% (4 of 10 patients) and 0% (0 of 18 patients), respectively. Moreover, the 20-wk immune-related progression-free survival rates were 78% (7 of 9 patients) and 11% (2 of 18 patients) for MMR-deficient CRC and MMR-proficient CRC, respectively. Additionally, patients with MMR-deficient non-CRC displayed responses similar to those of patients with MMR-deficient CRC[89]. Importantly, PD-1 blockade in patients with tumors with MSI has exhibited dramatic and durable responses, even in patients with colon cancer[89,90].

CONCLUSION

The goal of CRC immunotherapy is to induce efficient antigen-specific polyclonal $CD4^+$ and cytotoxic $CD8^+$ CTLs in patients. DCs are potent APCs that play a pivotal role in the induction of antitumor immune responses. Therefore, the use of DC-based immunotherapy for CRC patients is promising. However, the immune suppression synergistically generated from CRC and the tumor microenvironment continues to be a major hurdle. Here, we described the ability of DC-based therapeutic immunotherapies to activate antitumor immune responses in CRC patients. However, these strategies may require combination with immune-modulating agents to maximize antitumor immunity. The induction of antigen-specific polyclonal T cell activation may be

associated with the success of immune checkpoint therapeutic strategies. The combination of DC-based immunotherapy and simultaneous blockade of multiple immune checkpoints may have the potential for clinical benefit and should be evaluated[91].

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TOPIC HIGHLIGHT

2016 Hepatitis B virus: Global view

Precore/core region mutations of hepatitis B virus related to clinical severity

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Abstract

Despite the availability of an effective vaccine, hepatitis B virus (HBV) infection remains a major health problem, with more than 350 million chronically infected people worldwide and over 1 million annual deaths due to cirrhosis and liver cancer. HBV mutations are primarily generated due both to a lack of proofreading capacity by HBV polymerase and to host immune pressure, which is a very important factor for predicting disease progression and therapeutic outcomes. Several types of HBV precore/core (preC/C) mutations have been described to date. The host immune response against T cells drives mutation in the preC/C region. Specifically, preC/C mutations in the MHC class Ⅱ restricted region are more common than in other regions and are significantly related to hepatocellular carcinoma. Certain mutations, including preC G1896A, are also significantly related to HBeAg-negative chronic infection. This review article mainly focuses on the HBV preC/C mutations that are related to disease severity and on the HBeAg serostatus of chronically infected patients.

Key words: Hepatitis B virus infection; Precore/core mutations; Hepatocellular carcinoma; HBeAg serostatus; Disease severity

Core tip: The presence of several distinct types of mutations in HBV infections has been shown to contribute to the progression of liver disease in chronically infected patients. Although the relationships between single mutation types in the preC/C region and clinical severity have rarely been studied to date,

it was recently reported that some preC/C mutations, particularly in the MHC class Ⅱ restricted region, are significantly correlated with hepatocellular carcinoma. Several preC/C mutations, including preC G1896A, are also related to HBeAg sero-negative status, which can affect the disease progression of chronic patients. Mutations such as I97F/L or P135Q, which inhibit core nucleocapsid formation, also contribute to disease progression by evading host innate immunity. In addition, the P5H/L/T mutation may lead to hepatocarcinogenesis by inducing the ER stress-ROS axis. In this review, we mainly focus on the clinical implications of the reported preC/C mutations.

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INTRODUCTION

Despite the availability of an effective vaccine, hepatitis B virus (HBV) infection remains a major public health concern in most countries, but particularly in endemic areas such as China and South Korea. Globally, there are more than 350 million people who are chronically infected with HBV, which annually causes over 1 million deaths due to serious liver diseases, such as cirrhosis and hepatocellular carcinoma (HCC)[1].

HBV is an enveloped Hepadnavirus belonging to the *Hepadnaviridae* family. HBV has an incomplete double-stranded DNA genome that is approximately 3.2 kb in length and contains 4 overlapping open reading frames (ORFs) encoding the polymerase (P), core (C), surface antigen (S), and X protein[2,3]. Based on an intergroup divergence of > 8% in its complete genome sequence, HBV strains are classified into 8 genotypes, designated A-H, which correlate strongly with the ethnicity of infected patients[4-8]. There is increasing evidence that specific HBV genotypes may play significant roles in causing different disease profiles during chronic hepatitis B (CHB) infection[9-12]. Notably, an extraordinary prevalence of the C2 genotype has been reported in South Korea[13-15]. This genotype is more prone to mutations and is associated with more severe liver disease and poorer antiviral responses compared to genotype B[16,17]. In actuality, several types of HBV mutations that are rarely, if ever, encountered in other areas have been found in South Korea. These mutations were demonstrated *via* molecular epidemiologic and functional studies to be related to the disease progression of chronic patients[14,15,18-34].

Over the past decade, increasing attention has been focused on variant HBV strains that contribute to the clinical severity of liver diseases, especially HCC. To date, certain mutation patterns of HBV, such as the precore (preC) mutation at nucleotide 1896 (G1896A) or the double mutation in the basal core promoter (BCP) region at nucleotides 1762 (A→T) and 1764 (G→A), have been widely studied in the context of clinical severity[35-42]. Recently, several types of naturally occurring mutations in the pre-surface antigen (preS), S and X regions (*i.e.*, the preS1 start codon deletion[28,43], the preS2 deletion[28], W4P/R in preS1[29,32], sW182* in S[28,31,44], and V5M in X[23]), which are related to clinical severity, have also been described.

HBcAg AND HBeAg STRUCTURE AND THEIR VARIANTS

The HBV C protein antigen (HBcAg), the major structural protein of the nucleocapsid, is 183 residues long, of which the N-terminal 149 residues are the assembly domain[45-47]. The preC/C ORF is transcribed and translated into a precore/core fusion protein. During entry into the endoplasmic reticulum (ER), 19 residues of the 29-residue preC region are cleaved off by a signal peptidase, generating a 22-kDa protein. When transported into the Golgi compartment, additional amino acids are removed from the C-terminus by intra-Golgi proteases to form the HBe antigen, leading to a final, heterogeneous secreted protein of 15-18 kDa[48-50]. The secreted HBeAg is regarded as a marker of productive infection in clinical practice. Although the biological function of the HBeAg remains unsolved, it has been suggested that the HBeAg may contribute to HBV replication and modulate the host immune system as a type of tolerogen[51-53].

Most preC/C mutations are generated during HBeAg seroconversion in chronic HBV infections[54-56]. Such mutations can affect HBeAg serostatus and antigenicity, HBV nucleocapsid structure and stability, and the packaging of pregenomic RNA into the nucleocapsid[57]. HBcAg is the principal target of the host immune response and especially of CD4 and CD8 T cell attack[58,59]. Thus, mutations in the preC/C region are mainly distributed in the MHC-restricted region and can induce persistent HBV infections[27,60,61].

Several mutations in the preC/C region have been described, and the most frequently reported mutations in the literature are listed in Table 1[27,61-80]. Except in cases of nucleotide deletions, most mutations in the preC/C region are point mutations[65,80]. Most of the reported mutations in the preC region are associated with reduced HBeAg levels or reduced HBV replication in patient sera. In the C region, mutations are mainly located in immuno-active regions (MHC class Ⅰ + Ⅱ) rather than in immuno-inactive regions.

preC/C MUTATIONS RELATED TO CLINICAL SEVERITY OR HBeAg SEROSTATUS

Certain preC/C mutations have been associated with significant virological or clinical events, such as the

Table 1 Mutations in the hepatitis B virus preC/C region reported by previous literatures

Regions	Type of mutation	Mutations				HBV genotype	MHC class	Ref.
		Amino acid	Changes	Nucelotide	Changes			
preCore	AS	1	M1L/T/I	1814-1816	A1814T/C, T1815C, G1816T/A	A		[69-72]
	AS	2	Q2Stop	1817	C1817T	A		[71, 73]
	Ins		Insertion TT	1821	1821-1825			[74]
	Ins		Insertion TT	1825	1825-1826			[71]
	Del		T deletion	1839	1839del			[72]
	Ins		Insertion T	1839	1839-1840			[71]
	AS	15	P15S	1856	C1856T			[75]
			NC	1858	T1858C	A/B/E		[76]
	AS	17	V17F	1862	G1862T	A1		[95]
	AS	26	W26R	1889	T1889A	B/C		[77]
	Ins	36	Insertion 36nt	1895	1895-1896			[78]
	AS	28	W28Stop	1896	G1896A	B/C/C2/D		[29, 61, 77, 79, 80]
	AS	29	G29D	1899	G1899A	C2		[29]
Core	AS	5	P5T/L/H	1913-1915	C1913A, C1914A/T, G1915A/C	B/C2	Ⅱ	[29, 61, 81]
			P5R	1914	C1914G	A/D		[79]
			NC	1915	G1915A/C, A1915T	B/C		[77, 82]
	AS	12	T12S	1934	A1934T	A/C/D	Ⅱ	[83]
	AS	21	S21H	1961-1962	T1961C, C1962A	A/C/D	Ⅰ	[68, 83]
	AS	27	V/I27I/V	1979	G1979A, A1979G	A/B/C/D	Ⅰ	[68, 77, 83]
			NC	1981	C1981A	A/D		[68]
	AS	32	D32N/H	1994	G1994A/C	C2	Immuno-inactive	[29]
	AS	34	NC	2002	C2002A	A/D	Immuno-inactive	[68]
	AS	35	S35L	2004	C2004T	A/D	Immuno-inactive	[68]
	AS	43	E43K	2027	G2027A	C2	Immuno-inactive	[29]
			NC	2029	G2029A	A/D		[68]
	AS	45	NC	2035	T2035A/G	A/D	Immuno-inactive	[68]
	AS	48	NC	2044	T2044C	B/C	Immuno-inactive	[77]
	AS	49	NC	2047	A2047C	A/D	Immuno-inactive	[68]
	AS	50	P50Y/H/A	2048-2049	C2048T/G, C2049A	B/C/C2	Ⅱ	[29, 84]
	AS	55	L55I	2063	C2063A	A/D	Ⅱ	[68]
	AS	59	I59F	2075	A2075T	A/C/D	Ⅱ	[83]
			NC	2077	T2077A/C	B/C		[77]
	AS	60	L60V	2078	C2078G		Ⅱ	[85]
			NC	2080	C2080A	A/D		[68]
Core	AS	64	E/K64D	2090-2092	A2090G, A2092T/C	A/C/D	Ⅱ	[68, 83]
	AS	65	L65V	2093	C2093G	A/D	Ⅱ	[68]
	AS	67	T67N	2100	C2100A	A/C/D	Ⅱ	[68, 83]
	AS	77	E77Q	2129	G2129C	B/C	Immuno-inactive	[84]
			NC	2131	A2131G	A/D	inactive	[68]
	AS	78	NC	2134	C2134T	B/C	Immuno-inactive	[77]
	Del		105nt deletion	2134-2238	2134-2238			[86]
	AS	79	NC	2137	A2137G/T/C	B/C	Immuno-inactive	[77]
	AS	83	E83D	2149	A2149T/C	C2	Ⅱ	[29]
	Del		105nt deletion	2150-2254	2150-2254			[86]
	AS	87	S87R	2161	C2161G	B/C	Ⅱ	[77]
	AS	89	NC	2167	T2167C	A/D	Ⅰ and Ⅱ	[68]
	AS	92	NC	2176	T2176C	B/C	Ⅰ and Ⅱ	[77]
	AS	95	L95I	2183	C2183A	A/D	Ⅰ and Ⅱ	[68]
	AS	97	I97F/L	2189-2191	A2189T/C, C2191T	A/B/C/C2/D	Ⅱ	[29, 77, 83, 85]
			NC	2191	C2191A/T	A/D		[68]
	AS	100	L100I	2198	C2198A	A/C/C2/D	Ⅱ	[29, 83]
	AS	101	NC	2201	T2201C	B/C	Ⅱ	[77]
	Del		130nt deletion	2204-2333	2204-2333			[86]
	AS	107	NC	2221	C2221T	B/C	Immuno-inactive	[77]

AS	113	E113Q	2237	G2237C	A/D	Immuno-inactive	[68]
AS	115	NC	2245	C2245T	B/C	Immuno-inactive	[77]
AS	117	NC	2251	G2251A	B/C	Ⅱ	[77]
AS	119	NC	2257	G2257A	A/D	Ⅱ	[68]
AS	120	NC	2260	G2260A	B/C	Ⅱ	[77]
AS	126	NC	2278	T2278A	A/D	Ⅱ	[68]
AS	130	P130S/T	2288	C2288T/A	B/C	Ⅱ	[74, 77]
		NC	2290	C2290T	B/C		[77]
AS	131	A131P/N/G	2291-2293	G2291C/A, C2292A/G, T2293C	A/C/C2/D	Ⅰ and Ⅱ	[29, 83]
		NC	2293	C2293T	A/D		[68]
AS	134	NC	2302	A2302G	A/D	Ⅰ	[68]
AS	135	P135Q/S/A	2303-2304	C2303T/G, C2304A	A/B/C/D	Ⅰ	[61, 68, 83]
AS	142	NC	2326	A2326T	A/D	Ⅰ and Ⅱ	[68]
AS	145	NC	2335	A2335G	A/D	Ⅰ and Ⅱ	[68]
AS	149	V149I	2345	G2345A	B/C	Ⅰ and Ⅱ	[84]
AS	181	S181P/H	2441-2442	T2441C, C2442A	C2	Immuno-inactive	[29]
AS	182	Q182K/Stop	2444-2445	C2444A/T, A2445G	C2	Immuno-inactive	[29]

AS: Amino acid substitution; Del: Deletion; Ins: Insertion; MHC: Major histocompatibility complex; NC: No change.

failure to form a nucleocapsid, liver disease progression, or HBeAg seroconversion.

preC/C mutations related to HBeAg serostatus

As patients with HBeAg-negative CHB respond poorly to conventional interferon-alpha therapy, they should be treated differently from those with HBeAg-positive CHB[81-83]. Thus, preC/C mutation analysis can provide valuable information for the management of patients with HBeAg-negative CHB. Basal core promoter mutations (BCP, nt1742-1849) that suppress the production of preC mRNA at the transcriptional level may contribute to the defective synthesis of HBeAg *in vivo*[35]. However, the most frequently occurring mutations that are responsible for an HBeAg-negative hepatitis B profile are mutations that occur within the preC region and that inhibit translation of the protein due to frameshift mutations or premature stop codons (Table 1). Among the mutations reported thus far (Table 1), the mutation that is most often responsible for defective HBeAg secretion is a point mutation, namely a G to A transition at nucleotide 1896 (G1896A) that changes the 28th codon of preC from tryptophan (UGG) into a translational stop codon (UAG). The reduced HBeAg level can also have an important effect on HBV replication and can thereby influence liver disease progression, particularly in fulminant hepatitis and acute exacerbation of CHB[66]. Several studies have reported a positive association between the severity of liver disease and the occurrence of G1896A mutations[27,41,70]. However, it has also been reported that there is no correlation between this mutation and liver disease[72-74]. The discrepancy between different findings may be due to various factors, including HBV genotype, the geographical location or race/ethnicity of patients, host immune competence, and co-infection with other viruses, such as HIV or HCV[5,30,84-86].

Most published studies have reported that HBeAg-negative CHB due to the preC G1896A mutation is only common in non-A genotypes. In patients with genotype A, preC start codon mutations (A1814C/T, T1815C/A) that lead to a failure of HBeAg production have been frequently found[87], but such mutations are very rare in those infected with non-A genotypes. Recently, Mayaphi *et al*[61] reported that 24% of patients with sub-genotype A1 had preC start codon mutations, suggesting that this mutation, rather than the G1896A mutation, may contribute to HBeAg-negative CHB infection in patients with sub-genotype A1.

The G1862T mutation, which leads to a valine-to-phenylalanine amino acid substitution at residue 17 of the preC region, can affect the expression of HBeAg by interfering with signal peptidase cleavage[88]. This mutation prevails in HBV genotype A and particularly in sub-genotype A1; together with preC start codon mutations, G1862T is responsible for the HBeAg-negative serostatus and much lower viremia titers in patients infected with HBV genotype A. Moreover, Saha *et al*[77] recently reported that all tested HBV/A1 isolates from Eastern India harbored the G1862T mutation irrespective of HBeAg status, supporting the idea that this mutation might represent a natural variation in HBV/A1 rather than an adaptive mutation.

Mutations in the C-terminus of the preC/C region alter the biosynthesis, transportation, and secretion of HBeAg[73,79,89]. Such mutations lead to the cytoplasmic accumulation of the HBeAg proprotein (p22) in hepatocytes, resulting in decreased HBV replication in patient sera due to down-regulated HBV DNA replication and HBcAg capsid polymerization[73]. Recently, Wu *et al*[73] reported that, together with the G1896A mutation, the C2304A mutation, which causes a glutamine-to-proline substitution at residue 135 of HBcAg (P135Q), is a predictor of spontaneous HBeAg seroconversion following long-term immune-tolerance development

Table 2 Mutations in the hepatitis B virus preC/C region leading to change of HBeAg serostatus in chronic patients

Regions	Mutations				HBV genotype	MHC class	HBeAg serostatus (*P* value)	Ref.
	Amino acid	Changes	Nucleotide	Changes				
preCore	28	W28Stop	1896	G1896A	A/B/C/C2/D		N (n = 14) *vs* P (n = 3) (P = 0.004)	[27]
							eAg Seroconverters (n = 14 of 29, 48.3%)	[73]
							N (43.4%) *vs* P (11.9%) (P = 0.001)	[72]
							N (61.5%) *vs* P (10.9%) with HIV/HBV ($P \leq 0.0001$)	[77]
Core	5	P5R	1914	C1914G	A/D	Ⅱ	N (23.4%) *vs* P (4.7%) (P = 0.001)	[72]
	32	D32N/H	1994	G1994A/C	C2	Immuno-inactive	N (n = 2) *vs* P (n = 7) (P = 0.074)	[27]
	43	E43K	2027	G2027A	C2	Immuno-inactive	N (n = 1) *vs* P (n = 7) (P = 0.024)	[27]
	50	P50Y/H/A	2048-2049	C2048T/G, C2049A	C2	Ⅱ	N (n = 5) *vs* P (n = 0) (P = 0.020)	[27]
	131	A131P/N/G	2291-2293	G2291C/A, C2292A/G, T2293C	C2	Ⅱ	N (n = 4) *vs* P (n = 0) (P = 0.039)	[27]
	135	P135Q	2304	C2304A	B	Ⅰ	eAg Seroconverters (n = 18 of 29, 62.1%)	[73]
	181	S181P/H	2441-2442	T2441C, C2442A	C2	Immuno-inactive	N (n = 4) *vs* P (n = 0) (P = 0.039)	[27]

MHC: Major histocompatibility complex; N: Negative; P: Positive.

within chronic HBV genotype B-infected subjects. Based on a functional study, this mutation was significantly associated with increased cytoplasmic accumulation of the 22-kDa HBeAg proprotein (p22), decreased mature 17-kDa HBeAg (p17) secretion, and a decreased number of HBV capsid particles in Huh7 hepatoma cells, suggesting that this mutation may be associated with spontaneous HBeAg seroconversion in chronic HBV genotype B-infected patients *via* the decreased secretion of the 17-kDa mature HBeAg (p17)[57,90-92].

Recently, we reported that a total of 5 preC/C mutations (G1896A in preC plus 4 in the C region, namely E43K, P50A/H/Y, A131G/N/P and S181H/P) were found to be significantly related to HBeAg serostatus in chronic patients infected with sub-genotype C2[27]. Of those, interestingly, 2 mutations (D32N/H, and E43K) were related to the HBeAg-positive serostatus that was first introduced in the report. These 2 mutations were not located in the regions encoding T or B cell epitopes, suggesting that they may be induced by mechanisms other than immune evasion. Notably, none of the above-described 5 preC/C mutations that were related to HBeAg-negative serostatus was significantly related to HCC, although the G1896A mutation tended towards association with HCC (P = 0.093). These findings raise questions regarding the actual pathogenetic implications of HBeAg-defective mutants. Prospective studies on mutations in the preC/C region and their molecular mechanisms as they relate to the progression of liver disease would provide a better understanding of the clinical relevance of preC/C mutations in relation to HBeAg-negative serostatus. The preC/C mutations that have been reported to cause a change in the HBeAg serostatus of CHB patients are summarized in Table 2.

HBcAg mutations related to clinical severance

As HBcAg is the principal target of the host immune response and particularly of CD4 and CD8 T cell attack, the mutations in this region are mainly distributed in the MHC restricted region and may induce persistent HBV infection[58,59,78]. Indeed, a positive relationship between the frequency of HBcAg and the progression of liver disease has been reported[93-96]. We recently reported that the mutation rate in the MHC class Ⅱ restricted region (M2RR) (2.7% *vs* 1.9%, P = 0.024), but not in the MHC class I restricted region (M1RR) (2.4% *vs* 1.8%, P = 0.3), was significantly higher in HCC patients infected with sub-genotype C2 than in non-HCC patients with the same HBV genotype[27]. Furthermore, the difference between HCC and non-HCC patients with respect to the hotspot region in the M2RR (residues 81-105) was also pronounced (5.6% *vs* 2.6%, P = 0.002). In genotype A1-infected patients, HBcAg mutations were also most commonly found in the M2RR, suggesting that mutations in the M2RR of HBcAg that aid in immune escape can contribute to persistent HBV infection and influence disease progression[97].

We recently introduced 5 mutations in the HBcAg region (P5H/L/T, E83D, I97F/L, L100I and Q182K/Stop) that have been significantly associated with HCC patients compared to patients at other stages of the disease, such as liver cirrhosis (LC) and chronic hepatitis (CH)[27]. Notably, 4 of the 5 HCC-related HBcAg mutations, namely P5H/L/T, E83D, I97F/L and L100I, were located in the M2RR. I97F/L, which was previously known to lead to defects in HBcAg assembly[98,99], was most frequently found in HCC patients (13/35 HCC patients, 37.1%). The frequency of this mutation (16%) was also higher among sub-genotype A1 variants in South Africa[61]. Intrahepatic

Table 3 Mutations in the hepatitis B virus preC/C region related to clinical severance

Regions	Mutations				HBV genotype	MHC class	Clinical significance (*P* value)	Ref.
	Amino acid	Changes	Nucleotide	Changes				
preCore	28	W28Stop	1896	G1896A	C2		HCC (n = 12) *vs* Mild (n = 5) (P = 0.093)	[27]
					A/D		HCC (P < 0.05), FHF (45.0%) *vs* AVH (17.4%) (P = 0.038)	[72]
Core	5	P5T/L/H	1913-1915	C1913A, C1914A/T, G1915A/C	C2	Ⅱ	HCC (n = 5) *vs* Mild (n = 0) (P = 0.02)	[27]
		NC	1915	G1915A/C	C3		HCC (P = 0.020)	[70]
		P5R	1914	C1914G	C4		HCC (32.2%) *vs* ASC (5.0%)/ CHB (7.8%)/LC (10.3%) (P < 0.05)	[72]
	78	NC	2134	C2134T	C5	Immuno-inactive	HCC (P = 0.001)	[70]
	83	E83D	2149	A2149T/C	C6	Ⅱ	HCC (n = 4) *vs* Mild (n = 0) (P = 0.039)	[27]
	92	NC	2176	T2176C	C7	Ⅰ and Ⅱ	HCC (P = 0.139)	[70]
	97	I97F/L	2189-2191	A2189T/C, C2191T	C8	Ⅱ	HCC (n = 13) *vs* Mild (n = 4) (P = 0.024)	[27, 73, 93]
	100	L100I	2198	C2198A	C9	Ⅱ	HCC (n = 6) *vs* Mild (n = 1) (P = 0.046)	[27]
	107	NC	2221	C2221T	C10	Immuno-inactive	HCC (P = 0.001)	[70]
	115	NC	2245	C2245T	C11	Immuno-inactive	HCC (P = 0.007)	[70]
	130	P130T	2288	C2288A	C12	Ⅰ and Ⅱ	HCC (P = 0.022)	[70]
	182	Q182K/ Stop	2444-2445	C2444A/T, A2445G	C13	Immuno-inactive	HCC (n = 4) *vs* Mild (n = 0) (P = 0.039)	[27]

ASC: Asymptomatic S antigen carrier; AVH: Acute viral hepatitis; CHB: Chronic hepatitis B; FHF: Fulminant hepatic failure; HCC: Hepatocellular carcinoma; LC: Liver cirrhosis; MHC: Major histocompatibility Complex; NC: No change.

expression of HBcAg induced a robust IFN response in mice that facilitated control of the viral infection[100]. However, a recent study reported that the majority of mice that received a capsid assembly-deficient HBV mutant with the Y132A mutation in HBcAg failed to elicit the appropriate HBV-specific immune responses for eliminating hepatitis B surface antigenemia, suggesting that nucleocapsid formation is important for triggering a proper antiviral immune response, perhaps *via* the induction of innate immunity against HBV[101]. Thus, the I97F/L mutation, which is responsible for defective nucleocapsid assembly, may partially contribute to the progress of severe liver disease by failing to elicit a proper host immune response against HBV infection. We have previously reported that the lower level of HBV DNA in patients infected with mutated strains in preC/C region than in those with wild strains were found[27], suggesting preC/C mutations could lead to inhibition of HBV replication, generally. But, the identification of mutation types affecting HBV replication should also be done *via* functional study in the future.

Mutations in residue 5 of HBcAg (P5H/L/T or P5R) are reported to be significantly more frequent in HCC patients relative to a reference group, not only in Korean patients infected with sub-genotype C2[27] but also in Indian patients infected with genotypes A or D[72]. Recently, we demonstrated that P5H/L/T mutations in HBV genotype C2 can elicit the ER stress-ROS axis in hepatocytes[75]. This response then leads to inflammatory cytokine production, TGF-β secretion, apoptosis and HBsAg secretion, all of which are related to liver disease progression. The resulting prolonged inflammation, liver damage and increased HBsAg secretion induced by these mutations may contribute to the progression of liver disease in chronic patients.

Xie *et al*[70] recently reported that 5 mutation sites in the preC/C region, namely 1915, 2134, 2221, 2245, and 2288, were identified as statistically significant independent predictors of HCC survival by multivariate survival analysis. Of these, only the C2288A mutation in HBcAg (P130T) results in an amino acid change, while the other 4 mutations are silent. However, further validation in other populations and functional studies will be required to elucidate the mechanism by which these mutations, particularly the 4 silent mutations, affect HCC progression. The preC/C mutations that have been previously reported as associated with liver disease progression in chronic patients and particularly in HCC patients are summarized in Table 3.

CONCLUSION

Mutations in the preC/C region can affect HBeAg serostatus, HBV replication, nucleocapsid formation, and even pgRNA encapsidation[27,45,48,49,74]. Despite disparities in the infecting genotypes and in patient factors such as co-infection with HIV and patient ethnicity and geographical location, several preC/C mutation types that affect disease progression in chronic patients have been identified[61,77,78,87]. In general, mutations such as G1896A, which decrease or cause a loss of HBeAg, may contribute to disease progression *via* persistent

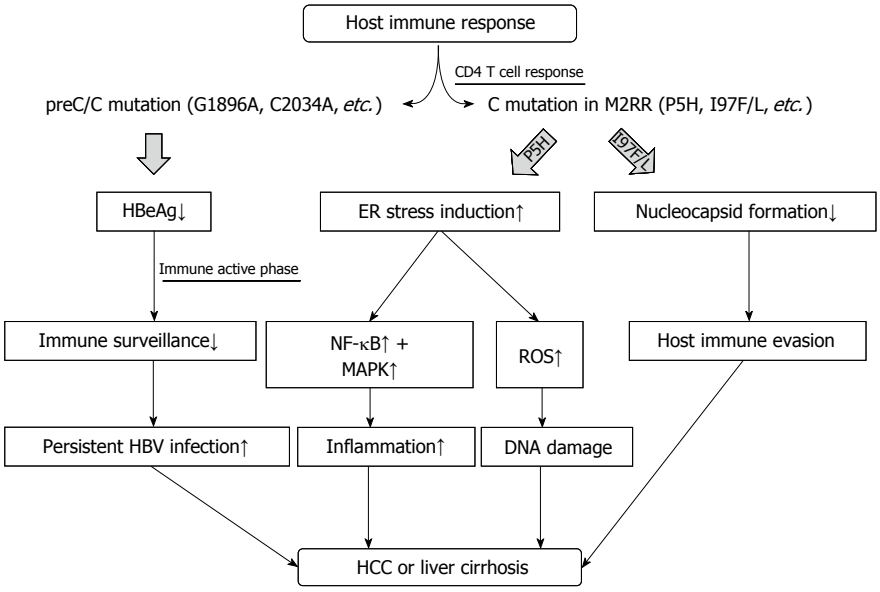

Figure 1 Schematic representation indicating role of mutations in the hepatitis B virus preC/C region in the disease progression of chronic patients. HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; ER: Endoplasmic reticulum; NF-κB: Nuclear factor kappa B.

HBV infection[21,102,103]. Immune-escape mutations in the M2RR of HBcAg, particularly in the hotspot region comprising amino acid residues 81-105, may contribute to persistent HBV infection that is related to disease progression[53,73,79,104]. Mutations such as I97F/L and P135Q, which inhibit core nucleocapsid formation, may also contribute to disease progression by evading host innate immunity[27,73,98,99]. The P5H/L/T mutation was shown to lead to hepatocarcinogenesis by inducing the ER stress-ROS axis (Figure 1). However, to better understand the clinical relevance of preC/C mutations, large, worldwide prospective studies on the clinical relevance of the different types of preC/C mutations are required, as are functional studies that relate the mutations to liver disease progression.

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REVIEW

Post-ablation surveillance in Barrett's esophagus: A review of the literature

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Abstract

Barrett's esophagus (BE) is a pre-malignant condition affecting up to 15% of patients with gastroesophageal reflux disease. Neoplastic Barrett's mucosa is defined as harboring high grade dysplasia or intra-mucosal cancer, and carries a high risk of progression to esophageal adenocarcinoma. The rising incidence of Barrett's lesions along with the high morbidity of surgical approaches has led to the development of numerous validated endoscopic techniques capable of eradicating neoplastic mucosa in a minimally invasive manner. While there has been widespread adoption of these techniques, less is known about optimal surveillance intervals in the post-therapy period. This is due in part to limitations in current surveillance methods, questions about durability of treatment response and the risk of subendothelial progression. As we are now able to achieve organ sparing eradication of superficial neoplasia in BE, we need to also then focus our attention on how best to manage these patients after eradication is achieved. Implementing optimal surveillance practices requires additional understanding of the biology of the disease, appreciation of the limits of current tools and treatments, and exploration of the role of adjunctive technologies. The aim of this article is to provide a comprehensive review of current literature surrounding post-ablation surveillance in neoplastic BE.

Key words: Barrett's esophagus; Endotherapy; Post-ablation surveillance; Neoplastic; Radiofrequency ablation; Endoscopic mucosal resection

Core tip: Hybrid endotherapy has become common practice for neoplastic Barrett's esophagus with many studies supporting its efficacy. There are limited data and recommendations on appropriate intervals of endoscopic surveillance in the post-therapy period. The purpose of this paper is to review the literature regarding

endoscopic surveillance following current endotherapy strategies for neoplastic Barrett's esophagus, discuss the deficiencies of current surveillance protocols, as well as to comment on the potential role of emerging modalities for monitoring disease progression in the post treatment setting.

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INTRODUCTION

In the United States, Barrett's esophagus (BE) is currently defined as the presence of endoscopically recognizable columnar mucosa in the esophagus, which is confirmed to have intestinal metaplasia in mucosal biopsy specimens that should be designated as at or above the gastroesophageal junction (GEJ). This definition has evolved in the guidelines over time as more data regarding risk for cancer progression has been discovered[1-5]. It is a condition that affects 5%-15% of patients with gastroesophageal reflux disease and approximately 2% of the total population[5,6]. It is believed to develop partly in response to acid exposure in the distal esophagus causing cellular changes that have the potential to form dysplasia or even cancer. Non-dysplastic BE progresses to cancer at a rate of 0.18%-0.3% per person per year based on robust population and cohort studies[7-10]. In the presence of high grade dysplasia (HGD), this risk of cancer is increased to the order of 15% per year[3,6,11]. While it has been demonstrated that non-dysplastic lesions can be safely monitored with surveillance endoscopy, the presence of neoplastic lesions hastens the need for interventions that eradicate dysplasia and lower both mortality and progression to cancer[4,11-14]. The rise in the incidence of esophageal adenocarcinoma in recent decades without significant improvement in patient outcomes has prompted the development of an array of endoscopic therapies for neoplastic BE. Following endoscopic therapy however, a paucity of guidelines exist for post-treatment surveillance and the optimal follow up interval is unknown[1-5]. The purpose of this paper is to review the literature regarding endoscopic surveillance following current endotherapy strategies for neoplastic BE, discuss the deficiencies of current surveillance protocols, as well as to comment on the potential role of emerging modalities for monitoring disease progression in the post treatment setting.

DYSPLASIA, ENDOTHERAPY, AND RECURRENCE

Dysplasia in BE is classified into non-dysplastic BE, low grade dysplasia (LGD), indeterminate for dysplasia, HGD, and intra-mucosal cancer (IMC). Often times HGD and IMC are combined into a category of neoplastic BE, with further sub-classifications based on depth of invasion. Recent analyses have concluded that endotherapy is equally as effective at achieving remission in high grade mucosal lesions as esophagectomy, but with less morbidity and fewer complications[4,14,15]. In the realm of endotherapy, both tissue acquiring and tissue damaging modalities exist and are often used in concert. Visible lesions are an indication for endoscopic mucosal resection (EMR) which can be used to confirm a diagnosis and allow appropriate staging[1-2,16-18]. Radical EMR has also been associated with higher complication rates, with strictures occurring in up to 37% in one cohort[16,18]. Photodynamic therapy was initially utilized to ablate Barrett's neoplasia based on early randomized trials demonstrating a reduction in cancer progression and elimination of dysplasia *vs* proton pump inhibitor alone[1,19]. However, this technique has also been shown to yield higher rates of residual buried metaplasia, recurrence of dysplasia, inability to ablate non-dysplastic lesions, and more frequent adverse effects[3,15,20]. Cryotherapy is a technique that has been used in smaller cohorts, however longitudinal randomized controlled data regarding outcomes are still needed[1-4,21]. In the last decade, circumferential radiofrequency ablation (RFA) has become one of the most commonly used endoscopic ablative therapies[4]. RFA is typically combined with focal EMR of visible lesions, and this hybrid method has produced high (> 90%) rates of eradication and a durable response up to 5 years post-treatment[12,22-25]. There is also emerging data that hybrid therapy in patients with LGD can decrease rates of progression to HGD and IMC by up to 25% with an acceptable safety profile when compared to optimal surveillance alone[13]. Proton pump inhibitors remain the standard of care in medical management regardless of whether endotherapy is pursued[1-4]. Their use decreases acid exposure in the distal esophagus and is thought to prevent the cellular changes that lead to the development of dysplasia and cancer, although this relationship has never been definitively proven[5,26,27].

Despite its high rate of eradication of dysplasia, there remain concerns about durability of response and recurrence patterns following hybrid endotherapy[20,23,25,28,29]. Multiple studies have demonstrated that recurrence of intestinal metaplasia and progression to cancer still happen in the post-treatment period[30-33]. One meta-analysis quotes recurrence rates of 11% following endotherapy with complete eradication of neoplastic lesions[14]. Most gastroenterologists agree with continued surveillance, but there still remains significant variability among endoscopic follow up in practice due to both patient and physician factors[4,15,34]. Part of this has been due to rapid advances in technology causing a shifting landscape

of ablative therapies, and the lack of large, high quality randomized controlled trials. The adequacy of current surveillance methods has also been called into question on numerous fronts. Sampling error, inter-observer variability, biopsy depth, properties of neosquamous epithelium and buried metaplasia, and metachronous lesions all provide challenges to the standard of targeted and four quadrant biopsies. The cost-effectiveness of post-ablation surveillance and new imaging technologies to detect buried intestinal metaplasia are also items gaining attention in the literature, as the financial burden of healthcare continues to grow[6,12,35]. All of these reasons highlight the need for evidence based protocols to guide surveillance in the post-treatment period.

CURRENT GUIDELINES FOR DYSPLASIA SURVEILLANCE AND PRACTICE TRENDS IN THE POST-ABLATION PERIOD

Endoscopic surveillance with four quadrant biopsies and targeted sampling of visible irregularities is the current standard of practice for patients diagnosed with BE. This technique has been implemented largely based on the assumption that earlier detection of dysplasia and treatable cancers will reduce deaths from esophageal adenocarcinoma and prolong survival[2,5]. A majority of the available evidence suggesting decreased mortality from surveillance has been retrospective to date[1-3]. Guidelines suggest that non-dysplastic BE can be followed with surveillance endoscopy every 3-5 years and targeted four quadrant biopsies every two centimeters (low quality of evidence). Most professional organizations recommend more aggressive surveillance intervals immediately following a diagnosis of dysplasia, based on the accelerated risk of developing esophageal adenocarcinoma. The presence of indeterminate for dysplasia or LGD calls for repeat endoscopy after 6 mo and, if confirmed, surveillance annually with biopsies every 1-2 centimeters (moderate quality of evidence). HGD/IMC requires targeted four quadrant biopsies every centimeter (low quality of evidence) with repeat endoscopy at 3 mo provided no ablative therapies or resection are initially pursued, in concert with consideration of further imaging and possible surgical consultation[1-4].

However, the frequency and duration of optimal surveillance following endoscopic therapy is less clear. Post-ablation surveillance is not discussed in the most recent position statement from the American Gastroenterological Association on the management of BE[2]. The American College of Gastroenterology guidelines suggest (grade D recommendation) that patients should be followed with biopsies in the area of prior Barrett's mucosa at intervals appropriate for their prior grade of dysplasia until there is "reasonable certainty of complete ablation" on at least three consecutive endoscopies. They go on to say that periodic surveillance is then recommended but there is insufficient evidence to offer specific time intervals[1]. The American Society for Gastrointestinal Endoscopy (ASGE) guidelines state that optimal surveillance intervals after ablation are unknown, however their authors recommend endoscopy every 3 mo for the first year following ablation, every 6 months in the second year, and annually thereafter (no associated level of evidence)[3]. One recent survey of 42 expert endoscopists found that all are performing post-ablation surveillance, and most are following the intervals suggested by the ASGE guidelines, with only a minority routinely ordering other imaging studies such as endoscopic ultrasound (EUS) and computed tomography for further staging[15].

DEFICIENCIES OF CURRENT SURVEILLANCE PROTOCOLS

Defining recurrence

Even though most experts agree that surveillance is beneficial following endoscopic treatment of neoplastic BE, there are deficiencies in the current surveillance process that cast doubt on our ability to reliably detect recurrence and progression of disease. First there is a lack of standardization in terminology when discussing disease recurrence. Most studies on RFA acknowledge that it often takes multiple sessions to achieve complete eradication of dysplastic lesions, but there is variability in the definition of complete eradication. Some studies count a single endoscopy free of visible and histopathologic findings of dysplasia adequate for achieving remission, whereas others require multiple consecutive endoscopies[1,22,25,36]. Often times, intestinal metaplasia is found incidentally on random biopsy of neosquamous epithelium following circumferential RFA of high grade lesions, and its implication on prognosis and the need for maintenance RFA treatment is uncertain[12,29,37-39]. When such areas are found and touched up, it is unclear if this constitutes residual metaplasia that was insufficiently treated, if it represents true recurrence of the parent lesion, or if it is a metachronous lesion that may be genetically independent with unknown malignant potential.

Sampling error and white light endoscopy

Furthering the difficulty in characterizing recurrence, is the fact that studies to date have utilized traditional white light endoscopy to identify areas with visible changes for targeted forceps biopsy, along with random four quadrant biopsies most commonly following the Seattle protocol[3]. Unfortunately, a random biopsy approach can be tedious, and does not provide information from a significant portion of the esophageal mucosa. Visible detection of the recurrence of intestinal metaplasia following ablation may also be insufficient with current imaging techniques. It has

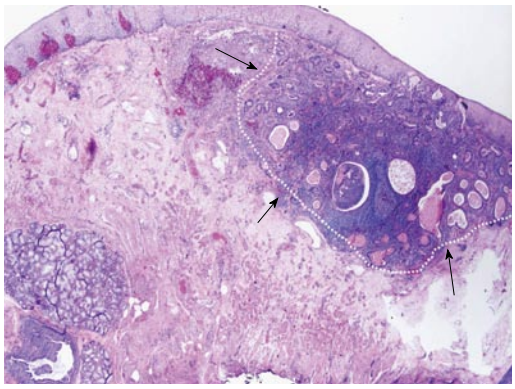

Figure 1 Subsquamous progression on endoscopic mucosal resection specimen. Outlined is an area of adenocarcinoma buried underneath squamous epithelium found on surveillance endoscopy, highlighting the potential danger of buried intestinal metaplasia.

been shown that endoscopic evaluation of the neo-squamocolumnar junction, even with the use of narrow band imaging, has limited sensitivity and specificity (sensitivity 65%-71%, specificity 37%-46%) for detecting biopsy confirmed intestinal metaplasia[40]. In addition, endoscopy and histology do not always tell the full picture of genetic changes that may be present in normal appearing neosquamous tissue, predisposing certain areas to tumorigenesis[33]. The heavy reliance upon visual inspection and random biopsies in our literature to date introduces inherent sampling bias, and has the potential for missing lesions not evident to the endoscopist, altering true rates of remission and recurrence[30]. The methodological weakness in this surveillance technique may in part limit our ability draw accurate conclusions on recurrence rates.

Buried metaplasia and adequacy of pinch biopsies

Buried intestinal metaplasia following ablation techniques has become recognized as an increasingly common phenomenon that may be underestimated by studies using pinch biopsies for surveillance. EMR and biopsy specimens can vary significantly in their final diagnosis, raising concern that surface biopsy findings do not accurately reflect the nature of a given lesion[18,32,37]. Unfortunately, EMR is not feasible in all areas where metaplasia is found on random sampling of the overlying neosquamous epithelium. Numerous studies have confirmed the existence of so-called subsquamous islands of intestinal metaplasia and dysplasia buried beneath normal and post-ablation epithelium[20,31-32,38,41]. Esophagectomy specimens have yielded rates of buried intestinal metaplasia as high as 71% confirmed by optical coherence tomography (OCT) and surgical pathology[42]. One study performing complete EMR for eradication of BE revealed subsquamous lesions with HGD or IMC in 21% of specimens prior to any tissue damaging therapy[32]. A systematic review of buried metaplasia following RFA yielded positive findings in only 0.9% of specimens on follow up biopsy, a rate much lower than for prior ablative techniques[20]. This is an encouraging finding for the use of RFA, but also raises concern over the adequacy of biopsy depth in post-RFA neosquamous epithelium. While it is known that the presence of lamina propria in a biopsy indicates adequate tissue purchase to sample the majority of mucosal lesions, this can often be difficult in the esophagus due to technical limitations[39]. Neosquamous epithelium may also be more fibrous, and limit the ability to achieve adequate biopsy depth. Three major studies looking at the depth of biopsies following photodynamic therapy or RFA ablation showed no difference between the number of pre-treatment and post-treatment biopsies containing lamina propria, despite their absolute percentages varying widely[29,43,44]. The clinical relevance of buried metaplasia remains in question due to a high prevalence and limited research into its malignant potential[38]. However, at least 8 cases of buried neoplasia have been reported in the literature to date, making the concept of subsquamous progression (Figure 1) a legitimate concern[31,38,42,45]. The possibility that endoscopically normal appearing mucosa may be harboring synchronous or metachronous lesions with cancerous potential beyond the reach of random biopsies creates a need for more precise methods of detection.

Histologic preparation and inter-observer variation

Appropriate post-ablation surveillance also relies on the proper handling and interpretation of biopsy specimens. The histopathological diagnosis of BE varies between American and British societies, with the American Gastroenterological Association still requiring goblet cell identification for confirming the diagnosis[2,37]. In practice, these stringent guidelines can be difficult to meet due to the small amount of tissue obtained and inability to sample the entire area of suspected Barrett's mucosa. Every step in biopsy processing is important to give the pathologist and endoscopist an accurate picture of the tissue they are sampling. The angle of the biopsy and how it is presented on the slide can impact whether glands appear buried or exposed to the surface, which has implications for their degree of acid exposure and malignant potential[29,37,38]. There is also a spectrum of dysplasia inherent in all biopsies that must be condensed into a single categorization, and this labeling has a large impact on surveillance intervals and therapy recommendations[37]. In the post-ablation population, regenerating tissue has a tendency to appear dysplastic due to inflammation and mesenchymal changes, and differentiating the level of dysplasia may be more difficult[37]. This can lead to over-calling of dysplasia, particularly in the community setting, to decrease the number of false negatives and prevent missing a cancer. Significant inter-observer variability also exists between pathologists, so confirmation with at least one expert gastrointestinal pathologist is recommended[1-4]. Research is ongoing to find supplemental genetic markers that can

reliably identify dysplastic changes that predispose an individual to developing cancer[33,46,47].

Durability of response and recurrence patterns

Effective eradication of intestinal metaplasia and IMC is believed to be possible by both tissue acquiring and tissue damaging methods[18,24,48-50]. Patient factors such as age and gender, characteristics of Barrett's lesions themselves, and the type and quality of endotherapy have been implicated in the ease of eradication and durability of response[27,36,51-53]. Effective post-therapy surveillance guidelines would require accurate information about durability of response, rates of recurrence, and patterns of recurrence for each available treatment option. Regarding hybrid EMR/RFA therapy, Shaheen *et al*[12] prospectively evaluated 127 patients with dysplastic BE demonstrating rates of eradication above 90% at 3 years based on white light endoscopy with targeted and surveillance biopsies. Even when factoring in patients lost to follow up, intestinal metaplasia was eradicated in 83% and dysplasia in 85% of cases. Phoa *et al*[23] looked at remission of neoplastic lesions 5 years following focal EMR and serial RFA in a 54 patient cohort using EUS and neosquamous resection to detect recurrence. They showed that 90% sustained complete eradication of neoplasia and intestinal metaplasia, with both neoplastic recurrences occurring near the 5 year cut off. In a multi-center review, Gupta *et al*[25] reported rates of recurrence using surveillance biopsies at 1 and 2 years as 20% and 33% respectively, with a majority being non-dysplastic. In general the literature supports a rate of recurrence between 5% and 30%, with high variability owing to differing methods for detecting recurrence, inclusion or exclusion of the GEJ, and different periods of follow up. Durability studies to date are limited by the methods used to evaluate for recurrence (*i.e.,* random biopsies) and likely underestimate its true prevalence, however the clinical relevance of buried intestinal metaplasia remains of some debate due to altered microenvironment and tissue properties[20,26,27,29,31].

Patterns and location of recurrence are another important factor surrounding the durability of endoscopic therapies that affects the need for surveillance. Studies have observed that synchronous and metachronous lesions are often present at various depths in the esophageal mucosa, and may not be adequately detected or treated by current techniques[6,28,32,37,41]. One study of EMR specimens found synchronous or metachronous lesions in as many as 28% of samples, and another using OCT found subsquamous metaplasia in 72% pre-RFA and 63% post-RFA[32,54]. The GEJ is a common culprit for harboring dysplastic lesions in patients with pre-existing BE. It has been speculated that this could be due to repeated acid exposure, difficulty in distinguishing the true location of the Z line on biopsy, and some feel that intestinal metaplasia represents a migration of cells from their origin at the GEJ. In one review of over 400 cases of BE following hybrid therapy with 37 recurrences, 19 involved the GEJ[25]. Vaccaro *et al*[6] also found that all cases of recurrent dysplasia in their cohort occurred at the GEJ in the absence of visible mucosal changes. For this reason, routine ablation of the GEJ has become a common practice and some also advocate surveillance and treatment of the superior gastric folds[40].

OTHER CONSIDERATIONS

Tissue acquiring vs non-tissue acquiring therapy

There is growing concern that tissue damaging therapies may select for dysplastic properties in the residual tissue and thereby predispose treated segments to the development of dysplasia over time. This may be related to certain clonal mutations being refractory to ablative therapy, and tissues containing these mutations would thereby be allowed to proliferate post-treatment creating a new, resistant dysplastic lesion[33,41]. Others have proposed that ablative therapies lead to *de novo* mutations in tumor suppressor genes p16 and p53 that can cause increased tumorigenesis[33]. Shaheen *et al*[48] showed that nearly 80% of post-RFA treated areas reverted to a neosquamous phenotype, however this does not account for genetic variants that may persist and predispose to neoplastic lesions buried under normal mucosa[31,41,45]. There has been some interest in more aggressive tissue acquiring therapies such as circumferential EMR and stepwise radical EMR to prevent these potential changes from occurring and propagating[18,50]. Epigenetic alterations are another area of evolving research into elucidating the genetic mechanism of BE progression and identifying at risk lesions. Loss of protection against hypermethylation of promoter regions around tumor suppressor genes is thought to play a role at multiple stages of tumorigenesis. These local hypermethylation events can vary in frequency across a neoplastic lesion, and less is known about their direct cause. It is unclear how various treatment strategies affect epigenetic modifications, but this area may evolve into one that can be applied clinically to identify patients at risk of recurrence after hybrid endotherapy[55-58]. Both genetic and phenotypic targets will likely be required in the future to truly understand a patient's risk profile[33,47]. Future research advances in the area of clonal mutations and epigenetics may support alterations in surveillance guidelines based on what type of therapy was rendered, however this remains controversial and requires additional research.

Complications of therapy and surveillance

As with any invasive procedure, complications of endoscopic surveillance and therapies must be considered when weighing risks and benefits in

discussion with patients. A recent meta-analysis comparing esophagectomy with endotherapy showed no difference in overall remission rate and mortality, with fewer major adverse events in the endotherapy group[14]. However, endotherapy also requires that the patient be willing to undergo some form of surveillance procedure(s), with the potential for additional therapies to be rendered on an as needed basis. Studies of decision-making have shown that patients' perceived risk of a procedure can vary widely based on their values, past experiences, personal relationships, baseline risk perception, mood, *etc*. When structuring the conversation of whether to treat endoscopically and how frequently to perform surveillance, providers must take into account the patient's subjective and objective risk perception, as some patients are willing to accept exceedingly high complication rates in order to have their disorder treated[34]. Alternatively, if a patient has an adverse event when therapy is rendered, they may be less amenable to post-treatment surveillance recommendations. Data on endoscopic complication rates and recommended surveillance intervals must be conveyed accurately to the patient using language they can easily comprehend. This can be difficult as these rates vary widely by institution, expertise of the endoscopist, and type of procedure. Pooled complication estimates for RFA or focal EMR followed by RFA vary from 5%-12% with the most common being esophageal stricture with or without dysphagia, bleeding, mucosal tears and dysrhythmias[13,25,48,49]. Some factors that have been associated with a higher risk of complications include length of BE segment, use of EMR in conjunction with RFA, and older age[25]. Reports on complete EMR also demonstrate rates of symptomatic strictures on the order of 37.8% and perforations around 1.9%[18]. Most adverse outcomes regardless of procedure type were easily treated endoscopically upon follow-up[13,25,48-50].

Cost effectiveness

In today's ever changing healthcare landscape, quality and cost control have become major considerations in population disease management. Endoscopic therapies have been found to be cost effective in patients with HGD compared to esophagectomy, and could add three quality-adjusted life years (QALY) at minimal cost[35,59]. In non-dysplastic BE or LGD, the cost-utility depends on the ability of endotherapy to durably eradicate the lesion. If an initial ablation could be definitive for these patients and obviate the need for further surveillance, then that could be considered cost-effective[25,35]. One analysis by Hur *et al*[59] compared different management strategies for patients with BE ranging from non-dysplastic to HGD. The model was based on a 50 year old individual being followed until age 80 or death, and compared surveillance with RFA once HGD developed *vs* initial RFA followed by surveillance endoscopy. For patients with no dysplasia, the incremental cost-effectiveness ratio for initial RFA *vs* surveillance was $205500 per QALY, assuming rate of progression of 0.12% per year. This was well above the study's willingness to pay threshold of $100000 per QALY. In patients with LGD, the incremental cost-effectiveness ratio for initial RFA *vs* a surveillance first strategy was $18231 per QALY assuming a rate of progression of 0.5% per year. Such complicated population modeling can be difficult to apply to individual patients, and there is inherent variation in the natural history of many LGD lesions[59]. Given the low rates of non-dysplastic progression, extended interval surveillance remains the recommended management strategy along with acid suppression at this time[1-4,11]. Unfortunately, over-surveillance is currently present in up to 2/3 of patients with non-dysplastic BE and presents a major area for improved health resource utilization[11]. Until further long term data are available, definitive cost-effective recommendations will remain difficult for the cohort of patients with indefinite for and LGD[11,13,35,59,60].

EMERGING SURVEILLANCE MODALITIES

Current surveillance recommendations remain dependent on biopsies of neosquamous epithelium as well as random mucosal sampling[1-4]. However, numerous advanced imaging modalities are now being applied to endoscopic techniques that have the potential for improving detection of recurrence and reducing sampling bias. Certain technologies show more progress than others in accomplishing this feat. In 2008, Savoy *et al*[61] showed that EUS provided little to no additional diagnostic value for patients with normal endoscopic biopsies and cross sectional imaging. It was primarily useful when abnormalities such as deeply invading tumors or extra-esophageal lymphadenopathy were found, and cannot differentiate between dysplastic and non-dysplastic mucosal lesions due to limited resolution[61,62]. Confocal laser microscopy (CLM), an endoscopic technique that allows real time microscopic analysis of surface features using fluorescent staining agents, has also been invoked to offer improvements in targeted biopsies during surveillance endoscopy[41,62-65]. It's diagnostic yield is limited to superficial lesions as deep as 250 μm, a depth insufficient to detect many sites of buried intestinal metaplasia, making its use in surveillance still incomplete[41,64,66]. Of note, one major randomized controlled trial adding CLM to standard white light imaging was stopped early due to a lack of difference between the experimental and control groups in detection of residual intestinal metaplasia, concluding that CLM did not add any additional diagnostic information[63]. Narrow band imaging (NBI) has also been touted as having the potential to detect patterns of intestinal metaplasia with reasonable

accuracy[67-69]. Many of the studies on NBI were unblinded and involved patients with long segment BE that was endoscopically easy to visualize. In patients with a normal appearing mucosa, particularly at the neo-squamocolumnar junction following RFA ablation, NBI showed a sensitivity of 71% and specificity of 37% for detecting residual intestinal metaplasia in one study. This same article also revealed that increasing confidence of the endoscopist in the diagnosis did not change the sensitivity and specificity values[40].

Of the emerging endoscopic techniques, OCT has shown promise for future diagnostic advancement. This ultra-high resolution device encompasses a fiber-optic probe that can be inserted into the accessory port of the endoscope with the ability to provide high quality volumetric images of the esophageal wall in real time using near infrared low coherence light[42,54,66,70]. It has the capacity to image to a depth of 1-3 mm with resolution on the scale of 3-5 μm[38]. One study by Cobb *et al*[42], utilized ultra high resolution OCT in fresh esophagectomy specimens of patients with HGD or esophageal adenocarcinoma. OCT was able to detect histologically confirmed subsquamous intestinal metaplasia, as well as differentiate between dysplasia and adenocarcinoma by imaging alone[42]. In another application of this technology, Tsai *et al*[66] performed OCT at the GEJ before and after RFA treatment and demonstrated that thinner lesions predicted higher success rates of ablative therapy. A depth of 333 μm or less was associated with a 92% sensitivity, 85% specificity, and 88% accuracy in predicting the absence of residual metaplasia at follow up endoscopy[38,66]. OCT has also been used to identify buried glands in pre and post-RFA specimens, demonstrating responses to treatment in real time[53]. It offers the potential for improved depth, a larger field of view, 3-D imaging, and reliable detection and differentiation of mucosal and submucosal abnormalities when compared to white light endoscopy with random biopsies[38,39,42,54,66]. Akin to other advanced imaging technologies, it is not immune to criticisms, including lack of standardized and validated criteria, added time to procedure, expense of the probes, variable endoscopic expertise, and limited speed of image processing[16,39,42,66].

Despite the fascination with endoscopic regression, many geneticists have argued for years that phenotype is only part of the story. Endoscopic improvement in the degree of visible abnormalities is no doubt important, but the genetic changes that underlie progression to cancer can persist in normal appearing mucosa[31,33,38,41,45]. The proportion of clonal abnormalities involving p16, p53 and chromosomal ploidy in a given lesion has been implicated in the genetic instability that causes progression to adenocarcinoma[47]. Some even feel these changes make mucosal segments inherently resistant to, and therefore clonally enhanced by, tissue damaging therapies[33,46,47]. Changes in such pro-tumorigenic loci have been implicated in both neosquamous epithelium as well as buried esophageal glands that may proliferate despite histologic normalization on biopsy in some cases[33,71]. We currently have limited ability to achieve real time molecular profiling that reliably detects mucosal genetic abnormalities during endoscopic intervention. It is hoped that with further study, novel genetic markers may become easier to detect in endoscopically normal mucosa, such that a hybrid genotypic and phenotypic approach to targeted surveillance and endotherapy might be achieved.

CONCLUSION

As we are now able to achieve organ sparing eradication of superficial neoplasia in BE, we need to also then focus our attention on how best to manage these patients after eradication is achieved. Implementing optimal surveillance practices requires additional understanding of the biology of the disease, appreciation of the limits of current tools and treatments, and exploration of the role of adjunctive technologies. Novel molecular targets combined with improvements in real time imaging of the epithelium and submucosal structures will likely continue to further our recognition of disease patterns and refine our understanding of recurrence. Patient centric models of surveillance and therapy may emerge as we learn more about the disease and inherent features that lead to increased morbidity and mortality. All of these advances must also be undertaken in a cost conscious way that will promote patient autonomy within the shared-decision making model. As we strive to reach a consensus on post-therapy surveillance guidelines, continued endoscopy with biopsies and vigilance of the endoscopist after eradication is paramount to achieving long term success of endotherapy in BE.

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REVIEW

Recent developments and innovations in gastric cancer

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Abstract

Gastric cancer has an important place in the worldwide incidence of cancer and cancer-related deaths. It can metastasize to the lymph nodes in the early stages, and lymph node metastasis is an important prognostic factor. Surgery is a very important part of gastric cancer treatment. A D2 lymphadenectomy is the standard surgical treatment for cT1N+ and T2-T4 cancers, which are potentially curable. Recently, the TNM classification system was reorganized, and the margins for gastrectomy and lymphadenectomy were revised. Endoscopic, laparoscopic and robotic treatments of gastric cancer have progressed rapidly with development of surgical instruments and techniques, especially in Eastern countries. Different endoscopic resection techniques have been identified, and these can be divided into two main categories: endoscopic mucosal resection and endoscopic submucosal dissection. Minimally invasive surgery has been reported to be safe and effective for early gastric cancer, and it can be successfully applied to advanced gastric cancer with increasing experience. Cytoreductive surgery and hyperthermic intraperitoneal chemotherapy were developed as a combined treatment modality from the results of experimental and clinical studies. Also, hyperthermia increases the antitumor activity and penetration of chemotherapeutics. Trastuzumab which is a monoclonal antibody interacts with human epidermal growth factor (HER) 2 and is related to gastric carcinoma. The anti-tumor mechanism of trastuzumab is not clearly known, but mechanisms such as interruption of the HER2-mediated cell signaling pathways and cell cycle progression have been reported previously. *H. pylori* is involved in 90% of all gastric malignancies and Japanese guidelines strongly recommend that all *H. pylori* infections should be eradicated regardless of the associated disease. In this review, we present innovations discussed in recent studies.

Key words: Gastric; Cancer; Endoscopic mucosal resection; Endoscopic submucosal resection; Minimally invasive surgery; Neoadjuvant chemotherapy; Human epidermal growth factor receptor 2

Core tip: Gastric cancers are distinguished from other cancers by their high mortality and morbidity. Many studies have been conducted to improve the quality of life and extend the survival rates of patients, and some of these studies are ongoing. Although promising developments have been made in recent years, the obtained results have limited reliability and benefits. We believe that significant improvements in the treatment of gastric cancer will be developed according to the long-term results of ongoing randomized clinical trials.

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INTRODUCTION

Gastric cancer is in the fifth most common cancer worldwide but it has the third highest incidence of death[1]. Gastric cancer usually does not metastasize to the distant organs until the third stage, but it can metastasize to the lymph nodes during the early stages, which is an important prognostic factor. Metastatic lymph nodes are correlated with the depth (T level) of the cancer. The recurrence observed after a D2- lymph node dissection (LND) is different from the recurrence observed after limited surgery, and locoregional recurrence can occur in most patients who undergo limited surgery. In addition, a minority of patients without perigastric lymph node metastasis can skip metastasis to distant lymph nodes[2,3]. The CA19-9 value is associated with the number of metastatic lymph nodes, and elevated CA19-9 values are significantly correlated (P = 0.008) with the number of metastatic lymph nodes. This could be useful for selecting advanced gastric cancer[4]. Curative surgery for gastric cancer consists of the excision of the mesogastrium, which contains lymph nodes and the omentum, with adequate surgical margins. The Japanese Research Society for the Study of Gastric Cancer (JRSGC) standardized the lymph node dissection for gastric cancer.

According to the JRSGC, a gastrectomy without D2-LND can only provide palliation. D2-LND was used to extend the lymphadenectomy in the 1960's in Japan. Currently, a para-aortic lymphadenectomy is defined as an extended lymphadenectomy. However, a D2-LND is known as an extended lymphadenectomy in Western countries[5,6]. Innovations of gastric cancer therapies include revising the gastrectomy and lymphadenectomy margins; reorganization of the TNM classification; developments in the endoscopic, laparoscopic and robotic treatment of gastric cancer; and innovations in cytoreductive, neoadjuvant and targeted therapies.

REVISIONS FOR GASTRECTOMY AND LYMPHADENECTOMY FOR GASTRIC CANCER

The classifications of lymph nodes have been upgraded intermittently since their first publication in 1962. Lymph node groups were classified as N1-N2-N3-N4, according to cancer location, in the first English edition[7]. The groups were formed based on the incidence of lymph node metastasis and according to the cancer location and the survival rate. The lymph nodes in the "N" groups were upgraded periodically. For example lymph node "7" was originally located in the "N2" group. However, in the third English edition, it was included in the "N1" group. The lymph nodes were grouped into 4 main groups (N1-3 and M1) in the second English edition[8]. This classification was misunderstood such that "N1 and N2" lymph node dissections were thought to be equal to "D1 and D2" lymph node dissections in countries outside of Japan[9]. This definition did not fully coincide with the Japanese classification system determined according to tumor location. For example, if the cancer was located in the proximal part of the stomach, the left paracardial lymph node (No. 2) was defined as N1; if the cancer was located in the corpus of the stomach, the left paracardial lymph node (No. 2) was defined as N3, and if the cancer was located in the distal part of the stomach, the left paracardial lymph node (No. 2) was defined as M (metastatic). This confusion is based on the difficulty of defining the classification. This complex classification system changed in 2010[10]. "D" dissection types (D0, D1, D1+, D2) are defined according to the type of total or subtotal gastrectomy instead of the old classification system[11] (Table 1). This classification system was more practical and easier to understand than the others.

D0 dissection is performed less often than D1 dissection. D1 dissection is preferred for T1a cancers that are not suitable for endoscopic mucosal resection (EMR) or endoscopic submucosal dissection (ESD). In addition, cT1bN0, well differentiated, $\leqslant$ 1.5 cm cancers are suitable for D1 dissection. D1+ dissection includes cT1N0 tumors that are not suitable for D1 dissection (> 1.5 cm, poorly differentiated cancers). D2 dissection is suitable for the gastric cancers consisting of potentially curable T2-T4 and/or cT1N+ tumors. D2+ dissection involves removing the para-aortic lymph nodes in addition to the D2 lymph nodes.

Mesenteric vein lymph node dissection (No. 14v) is described as a part of the D2 dissection for distal gastric cancers in the previous edition of the guidelines. However, in the current edition, these lymph nodes are removed from the classification. Furthermore, removing the No.14v lymph nodes can be useful if apparent metastasis to the subpyloric lymph nodes (No. 6) occurs, and this dissection is called D2+No.14v. According to the latest guidelines, lymph nodes behind

Table 1 Lymph node dissections according to gastrectomy type for gastric cancer

Type of gastrectomy	Type of disection	Retrieved lymph node stations
Total	D0	Less than D1
	D1	No. 1-7
	D1+	D1 + No. 8a, 9, 11p[1]
	D2	D1 + No. 8a, 9, 10, 11p, 11d, 12a[1]
Distal subtotal	D0	Less than D1
	D1	No. 1, 3, 4sb, 4d, 5, 6, 7
	D1+	D1 + No. 8a, 9
	D2	D1 + No. 8a, 9, 11p, 12a
Pylor preserving	D0	Less than D1
	D1	No. 1, 3, 4sb, 4d, 6, 7
	D1+	D1+ No. 8a, 9
Proximal	D0	Less than D1
	D1	No. 1, 2, 3a, 4sa, 4sb, 7
	D1+	D1 + No. 8a, 9,11p[2]

[1]If the cancer has invaded the esophagus, the No. 110 lymph node must be removed in addition to D1+ dissection, and the No. 19, 20, 110 and 111 lymph nodes must be removed in addition to D2 dissection; [2]The No. 110 lymph node must be removed in addition to D1+ dissection.

the pancreatic head (No.13) must be dissected if the cancer has invaded the duodenum, and this dissection is defined as D2+ No.13. A prophylactic para-aortic lymphadenectomy is not recommended due to the increased number of postoperative complications and the reduced survival, according to a Japanese randomized clinical trial (RCT) (JCOG 9501)[12]. In the absence of direct invasion of the spleen and macroscopic splenic hilar lymph node metastasis, a splenectomy for dissection the splenic hilum (No. 10) and splenic artery (No. 11) lymph nodes is controversial. The results of RCT JCOG 0110 will provide guidance[13] on this matter.

DEVELOPMENTS IN THE TNM STAGING SYSTEM FOR GASTRIC CANCER

The TNM staging system is the gold standard for staging of all types of cancers. The depth of the cancer and number of the metastatic lymph nodes are the most important prognostic factors for curative gastric cancer surgery. Two major staging systems exist for gastric cancer. The first system is the Japanese Gastric Carcinoma Classification (JGCC) which is based on the location of the metastatic lymph node, and the second is the Union Internationale Contre le Cancer/American Joint Committee Cancer (UICC/AJCC) TNM staging system, which is based on the number of metastatic lymph nodes[14].

The TNM classification system was adapted to the JGCC in 2009 and called the UICC/AJC TNM staging system in the 7th edition. This system can be effective for evaluating the clinical and pathological data and for minimizing the stage migration phenomenon. The main principles of pT and pN, according to this new staging system, are shown in Table 2.

Table 2 Comparison of the sixth and the seventh TNM staging systems for the pT and pN stages

Tumor localization	6th TNM staging system	7th TNM staging system
Lamina propria or muscularis mucosa	T1	T1a
Submukoza	T1	T1b
Muscularis propria	T2a	T2
Subseroza	T2b	T3
Serozal invasion	T3	T4a
Adjacent organ invasion	T4	T4b
1-2 lymp node metastasis	N1	N1
3-6 lymp node metastasis	N1	N2
7-15 lymp node metastasis	N2	N3a
⩾ 16 lymp node metastasis	N3	N3b

Another important difference between sixth and seventh TNM staging systems is that M0 patients could have been classified as stage Ⅳ in the sixth edition. However, in the seventh edition, only M1 patients (positive peritoneal fluid and liver, lung, bone, or brain metastasis) are classified as stage Ⅳ. In addition, a stage ⅢC sub-group has been added (T4aN3M0, T4bN2M0, and T4bN3M0). Esophagogastric cancers that have not invaded the esophagus and that are below the Z line are included in the gastric cancer TNM staging system. Esophagogastric cancers that are located in the proximal 5 cm area or that have invaded the esophagus are included in the esophageal cancer TNM staging system[1,15].

Some authors have suggested that the UICC/AJCC TNM staging system can cause stage migration phenomenon[16]. Patients with less than 15 lymph nodes removed were not included in the N3 classification in the sixth edition of the TNM staging system. Stage migration phenomenon can be prevented because the presence of 7 or more metastatic lymph nodes is classified as N3 in the seventh edition. However, this issue is still controversial. The reduction of the stage migration has not yet been shown in the seventh edition of the UICC/AJCC TNM staging system[17]. In clinical practice, especially when considering adjuvant treatment, the true staging of gastric cancer is very important[18]. Additionally, after removing an insufficient number of lymph nodes and staging the gastric cancer according to the UICC/AJCC TNM staging system of these lymph nodes, the prognosis of patient will be poorer than expected. A new classification system that is based on the ratio of metastatic lymph nodes to the total number of lymph nodes removed (N ratio) has been proposed for more accurate staging of gastric cancer and a more reliable prognostic assessment[19-21]. However, this classification system is in the hypothetical stage. Determining the cut-off value and the fact that this system is only useful for patients with less than 15 lymph nodes removed are the main problems for N ratio staging. The N ratio staging system requires further study.

ENDOSCOPIC INTERVENTIONS FOR EARLY GASTRIC CANCER

Surgical resection has long been the primary treatment for gastric cancers. Minimally invasive surgery and endoscopic treatment modalities have been used with increasing frequency to prevent the mortality and morbidity caused by conventional surgery. With these new interventions, less invasive and less costly treatment protocols that do not have any negative impact on oncologic outcomes, preserve physiological functions, and improve the quality of life after surgery have been developed.

Different endoscopic resection (ER) techniques have been identified, and these can be divided into two main categories: endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD)[22-24].

Patients with very low risk for lymph node metastasis and local recurrence are ideal candidates for ER. Early gastric cancer (EGC) is a limited malignant lesion in the gastric mucosa and submucosa, regardless of lymph node metastasis, and has excellent survival rates with curative treatment[25]. However, despite the reported high long-term survival rate, 3% of mucosal cancers and 20% of submucosal cancers exhibit lymph node metastasis[26]. The first indications for ER (differentiated cancer, < 2 cm tumor, and lesions with no ulceration or lymphovascular invasion that are limited to the mucosa) were determined empirically[27]. The extended indications for ER are still being discussed.

Japanese and South Korean gastric cancer treatment guidelines recommend that extended indications for ER should not be used for routine clinical practice, only for clinical research, due to the lack of high level evidence regarding the curative effect of ER[11,28]. In addition, the guidelines also suggest that ER should be applied according to standard indications. However, some gastric cancer treatment guidelines [National Comprehensive Cancer Network (NCCN), the European Society for Medical Oncology (ESMO), the European Society of Surgical Oncology (ESSO) and the European Society of Radiotherapy and Oncology (ESTRO)] have suggested that obtaining negative horizontal and vertical margins with ER is adequate for the treatment of gastric cancers that are < 2 cm, are well/moderately differentiated, have no lymphovascular invasion and are not located under the submucosa[29].

We can assess to the high level of evidence of the efficacy and safety of ER with the results of randomized clinical trials that compare gastrectomy and ER. However, no randomized clinical trials have compared ER and gastrectomy. The initial information generated by compiling data from 12 institutions in Japan indicates that if negative horizontal and vertical margins are present, EMR is an effective and safe treatment[30]. According to these results, EMR has a 75.8% *en bloc* resection rate, a 73.9% complete resection rate, a 1.9% recurrence rate after complete resection, and a 99% gastric cancer-specific survival rate. Recently, in a matched cohort study that compared EMR and gastrectomy, no difference was observed in the complication rates in terms of survival and recurrence between the groups. The risk of metachronous gastric cancer was higher in the EMR group, but shorter hospital stays and lower costs were reported as the benefits of the EMR procedure[31].

The use of ER increased when ESD was applied, and higher curative resection rates than those produced by EMR were obtained. Although different results from various clinical centers were obtained, rates of 65%-100% for unblocked resection, 68%-95% for complete resection, 94%-100% for 5-year recurrence-free survival and 95%-100% for 5-year survival have been reported for ESD[32,33]. According to a meta-analysis examining 3548 EGC cases and comparing EMR and ESD, ESD produced higher unblocked resection rates (odds ratio: 9.69; 95%CI: 7.74-12.13), higher complete resection rates (odds ratio: 5.66; 95%CI, 2.92-10.96) and lower recurrence rates (odds ratio: 0.10; 95%CI: 0.06-0.18)[34].

In another meta-analysis, standard ESD criteria were compared to the extended ESD criteria. No differences in the overall survival rates were found between the ESD and extended ESD groups. However, a higher rate of complications was observed in the extended ESD group[35]. In a retrospective study, ESD was compared to gastrectomy, and similar oncological results were obtained. However, lower complication rates were observed in the ESD group[36]. Although the ESD procedure is considered adequate for many EGC patients, histopathological examinations have shown that in 5%-20% patients, the procedure is non-curative[37]. Due to the risk of lymphatic metastasis and non-standard presentations (deep submucosal invasion and the presence of lymphovascular invasion), surgical resection with a lymphadenectomy should be performed. Surgery is suggested in the presence of positive lateral surgical margins; however, ER, endoscopic ablation therapy or close monitoring are also feasible[38,39]. The oncologic efficacy of ER has not been supported by a high level of evidence because most recent studies have consisted of retrospective comparisons of non-homogenous groups[36]. In addition, the clinical studies were performed mostly in the South Korea or Japan, which have a 50% rate of EGC. In Western countries, EGC is performed at lower rates; therefore, ER has been applied at lower rates than in Japan or South Korea. Due to these reasons, the applicability of ER by endoscopists is limited[40]. Detecting the early stages of gastric cancer and more widespread use of ER modalities for selected indications will be possible with the implementation of standardized training modules in Western countries[22,41].

MINIMALLY INVASIVE SURGERY FOR GASTRIC CANCER (LAPAROSCOPIC AND ROBOTIC SURGERY)

Laparoscopic surgery

Minimally invasive surgery (MIS) has been increasingly performed due to new surgical tools and the development of techniques for gastric cancer surgery. MIS has some short-term and long-term advantages. MIS has been reported to be safe and effective for EGC, and it can be successfully applied to advanced gastric cancer (AGC) with increasing experience[42-44]. T1 gastric cancer, which has clinically been shown to exhibit perigastric lymph node involvement, and gastric cancer, which has no serosal and lymph node involvement, are expanded indications for MIS[45]. The laparoscopic assisted distal gastrectomy (LADG) was described for EGC in 1991[46]. LADG for EGC has shown short-term benefits, such as reduced intraoperative blood loss and providing early postoperative mobilization, in a meta-analysis of RCTs[47]. The short-term results of laparoscopic gastrectomy (LG) are favorable, but the long-term results for gastric cancer are still controversial. Despite the increasing use of laparoscopic surgery for gastric cancer, a low level of evidence exists. Six RCTs have compared LG and open gastrectomy (OG)[48-53]. Recently, Chen *et al*[54] reported a meta-analysis that included 7336 patients and 23 studies. In this meta-analysis, the 5-year survival and death related to the gastric cancer rates were compared between the LG and OG groups. The 5-year overall survival, recurrence and gastric cancer-related death rates were comparable for LG and OG. The authors suggested that, based on current information at the end of the study, LG provided oncologic safety for early and advanced gastric cancer surgery. LADG has been compared with the open distal gastrectomy (ODG) in some studies, and no significant difference has been found in the 3-year survival rates[55-57]. Choi *et al*[58] reported no significant differences in the overall survival and disease free survival rates over a long period. Zhang *et al*[59] also found no significant differences in recurrence rates between LG and OG for EGCs. Tang *et al*[60] published a review consisting of 32 independent studies that compared LG and OG. They reported less intraoperative blood loss, less pain, earlier return to mobilization, earlier return of bowel sounds and shorter hospital stay as benefits of LG and found no difference in mortality between LG and OG. In addition, they stated that the increased operation time is the only disadvantage of LG, which can be solved by developing surgical techniques.

Fewer lymph nodes were removed during the first applications of MIS than by OG[61]. However, the number of the lymph nodes removed became similar to that of OG as surgeons gained experience[62]. LG is defined as a safe, feasible procedure, especially for EGC, in many studies, and this statement is widely accepted[42,63]. The success of this method depends on factors such as the experience of the surgeon, surgeon's experience with laparoscopy, hospital volume and gastric cancer volume of the surgeon, and preoperative diagnosis. These factors have been found in many studies[42].

With the development of surgical instruments and the increasing experience of surgeons, efforts have been made to decrease the number of ports used for MIS and to develop a single incision technique[64]. However, carbon dioxide pneumoperitoneum, increased intra-abdominal pressure, prolonged operative time, less lymph node removal, port site metastases and technical issues are still problems for laparoscopic gastric cancer surgery[47,65]. MIS does not increase peritoneal spread and port site metastasis according to many studies[66,67].

The short-term results of MIS applications for AGC have been described in the literature[42,68]. Authors report that MIS is a viable option compared to OG for selected cases. Son *et al*[43] reported similar survival and recurrence rates for MIS and GC for T4a cancers. In a meta-analysis that compared OG with D2 dissection and LG with D2 dissection, similar overall survival and major complication rates were observed. However, less blood loss, less pain, reduced minor postoperative complications and shorter hospital stays were reported for the LG patients[69]. However, some experienced surgeons have suggested that current surgical instruments are not sufficient for D2 dissection during MIS for AGC, and they have published their oncological results[48,68,70]. Some ongoing RCTs (JOCG-0912, JLSSG-0901, KLASS-01, KLASS-02, and CLASS-01) are being performed to assess the feasibility of MIS in Korea, Japan and China[71-75].

Robotic surgery

Robotic technology has developed new tools for use in MIS during the past decade[42]. The first robot-assisted gastrectomy (RAG) was reported by Hashizume and Sugimachi in 2003[76]. RAG has been used for gastric cancer surgery to overcome the technical difficulties of LG[77]. RAG has potential technical advantages such as providing a three-dimensional image, articulated instruments, and allowing for precise movement. In addition, RAG has spread rapidly[42]. Compared to the LG, RAG provides better images and movements. RAG is more effective and safe than LG according to many experienced surgeons[78,79].

In a meta-analysis by Xiong *et al*[80], LG and RAG were compared regarding their effects on gastric cancer treatment. RAG produced less intraoperative blood loss and comparable mortality and morbidity rates. However, the operation time was significantly longer than that for LG and OG.

The potential advantages of RAG include facilitation of intra-corporeal anastomosis and allowing extended

Table 3 Ongoing multicentric studies of minimally invasive surgery

Country	Study	Subject
Japan	JCOG 0912 Phase Ⅲ	LG *vs* OG
South Korea	KLASS 01 Phase Ⅲ	LG *vs* OG
South Korea	KLASS 02-NCT01456598	LG *vs* OG (for AGC)
Japan	JLSSG0901 Phase Ⅱ-Ⅲ	LG *vs* OG (for AGC)
China	CLASS 01-NCT01609309	LG *vs* OG (for AGC)
South Korea	KLASS 03-NCT01584336 Phase Ⅱ	LG *vs* OG (for TG)
South Korea	NCT01309256	LG *vs* RAG

AGC: Advanced gastric cancer; LG: Laparoscopic gastrectomy; OG: Open gastrectomy; RAG: Robot asisted gastrectomy; TG: Total gastrectomy.

lymph node dissection. However, inconsistent results have been presented in the literature regarding this subject[42]. RAG would be useful for overcoming the challenges of traditional LG, but it has not provided the theoretical advantages of lymph node dissection[42]. RCTs involving RAG have not been reported, However, the recent meta-analyses are weak and include few patients[80,81].

The overall and major complication rates were similar to the short-term surgical results of the multicenter NCT01309256 study from Korea (11.9 *vs* 10.3 and 1.1% *vs* 1.1%, respectively). However, the operation costs (US $13432 *vs* US $8090, $P < 0.001$) and time (221 min *vs* 178 min, $P < 0.001$) were significantly higher for RAG[82].

ONGOING MULTICENTRIC STUDIES OF MINIMALLY INVASIVE SURGERY

The final results of the KLASS 01 Phase Ⅲ study for stage Ⅰ gastric cancer patients are expected to show the oncologic safety of the treatments. In the early results of this study, no significant differences were found between the LG and OG groups regarding mortality and morbidity. No significant difference between the MIS and OG groups regarding 3-year overall survival rates were observed according to the first results of the multicentric KLASS 02-NCT01456598 study. Phase Ⅱ and Ⅲ studies (JLSSG0901 trial; UMIN-000003420) are being conducted by the Japanese Laparoscopic Gastric Surgery Study (JLSSG) group to investigate the technical and oncologic safety of laparoscopic treatment. The feasibility and the oncological safety of laparoscopic treatment of AGC are being investigated by the Chinese Laparoscopic Gastrointestinal Surgical Study Group (CLASS) in the CLASS 01-NCT01609309 study. The ongoing phase 2 KLASS 03-NCT01584336 study is investigating the feasibility and safety of laparoscopic and open gastrectomy for stage 1 gastric cancer patients in Korea. In addition, the NCT01309256 study continues to compare RAG and LG (Table 3).

INTRAPERITONEAL CHEMOTHERAPY

Gastric cancer is a biologically aggressive tumor. The prognosis is poor even if curative surgery can be performed. For higher stages of stomach cancer, the most common form of invasion is peritoneal metastasis[83]. Almost all patients with positive peritoneal cytology progress to peritoneal carcinomatosis and die within the first two years of the disease[84]. The peritoneum is supported by the basal membrane of mesothelial cells and connective tissue. The blood- peritoneal barrier is located between the mesothelial cells and mesothelial capillaries. Few systemic chemotherapeutic agents can pass through this barrier. Additionally, intraperitoneal chemotherapy has less adverse effects and produces a higher dose in the intraperitoneal regions than systemic chemotherapy[85]. Intraperitoneal chemotherapy can be given preoperatively and during the early postoperative period (EPIC). Intraperitoneal chemotherapy, given preoperatively, is aimed to prevent micro metastasis, increase the chance of curative resection and perform a complete cytoreduction. EPIC is given as soon as the general condition of the patient has recovered after surgery. It is started during the period in which the minimal residual tumor load is present and before the residual cancer cells become hidden between fibrin deposits[86].

EXTENSIVE INTRAOPERATIVE PERITONEAL LAVAGES

Kuramoto *et al*[87] developed a treatment modality called "extensive intraoperative peritoneal lavage treatment" (EIPL), which aims to destroy the free cancer cells spreading into the peritoneum. After a curative resection is performed, the abdomen is washed with 1 liter of isotonic saline and aspirated. Then, this procedure is repeated 10 times. The aim of this method, which is called "Limiting dilution method", is to remove the free cancer cells in the peritoneum by washing with isotonic saline. A prospective randomized controlled study was performed that included 1522 patients with higher stage stomach cancer who had undergone curative resection (R0) and D2 dissection. Then, 88 patients with positive cytology and without peritoneal invasion (CY+/P-) were divided into 3 groups. Surgery alone was performed on for the first group. The second group was treated with intraperitoneal chemotherapy, and the third group was treated with EIPL+intraperitoneal chemotherapy. In the group given prophylactic intraperitoneal chemotherapy and intraoperative peritoneal lavage, the 5-year survival rate was markedly increased compared to the other group. The 5-year survival rates of each of the three groups were 0%, 4.6% and 43.8%, respectively. Standard prophylactic treatment against peritoneal metastasis has been reported as an effective treat-

ment modality. It is practical, can be performed in any situation, and does not extend the operation time. In the reported studies, prophylactic treatments used to prevent peritoneal metastasis in the early period has been shown to be promising[84,87-89].

CYTOREDUCTIVE SURGERY AND HYPERTHERMIC INTRAPERITONEAL CHEMOTHERAPY

Cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC) were developed as a combined treatment modality from the results of experimental and clinical studies[90]. In the 1990's Sugarbaker described the surgical method in detail. Complete cytoreduction must be performed before HIPEC is administered. Hyperthermia increases the antitumor activity and penetration of chemotherapeutics[91].

A meta-analysis of the results of 13 randomized studies (1648 cases) examined the benefits of adjuvant intraperitoneal chemotherapy after curative gastric cancer resection. It reported that patients who received intraperitoneal chemotherapy exhibited better survival[92]. Yang *et al*[93] reported median survival times of 11 and 6.5 mo, respectively, in their prospective randomized phase Ⅲ clinic study that compared the effects of CRS + HIPEC and CRS alone on 68 patients with gastric peritoneal carcinomatosis.

The survival time was increased to 13.5 mo after complete macroscopic cytoreduction (CC-0/1). Gill *et al*[94] summarized the data of 10 studies (n = 445), including one prospective controlled study, 3 retrospective case reports and 6 prospective case series, in which they found a median survival time of 15 mo (9.5-43.4 mo) for CC-0/1 patients. Additionally, the study results of pioneering authors such as Fujimoto, Sugarbaker, Glehen and Yonemura showed that the median survival time ranged between 11 and 16 mo in patients who underwent HIPEC with partial or complete cytoreduction[91,95]. Systemic chemotherapy produces a very limited survival benefit for patients who undergo CRS and HIPEC, increasing the life expectancy by 30%. This shows that systemic chemotherapy and HIPEC are more beneficial for patients who have been surgically treated. Koga *et al*[96] investigated the benefits of HIPEC as an adjuvant therapy for the prevention of peritoneal recurrence in a limited number of patients with gastric cancer and serosal invasion in a randomized clinical study. They found 3-year survival rates of 67.3% and 83% in patients with surgery plus HIPEC and in the control group.

Additionally, Hamazoe *et al*[97] found an increased survival rate in patients who underwent prophylactic HIPEC with high dose mitomycin C compared to the control group (64.2% *vs* 52.5% respectively). However, a randomized clinical study by Fujimoto, Fujimura and Yonemura showed that adjuvant HIPEC treatment decreased peritoneal recurrence and increased the survival rates of AGC patients[98-100]. Only one prospective randomized study showed that adjuvant HIPEC treatment produces no survival advantage[101]. The SRC and HIPEC multimodal combined treatment can only produce survival benefits for patients with gastric peritoneal carcinomatosis with well-defined boundaries. However, more detailed clinical studies are needed to determine the role of modern systemic chemotherapy[90].

NEO-ADJUVANT CHEMOTHERAPY FOR GASTRIC CANCER

Pre- and post-operative chemotherapy are accepted as the standard treatments for curable gastric cancers, except for the stage 1 gastric cancers, in Europe and England[102]. These results were concluded from the results of the Medical Research Council Adjuvant Gastric Infusion Chemotherapy (MAGIC) study[103]. In the USA and some Latin American countries, post-operative chemotherapy is the gold standard because it has narrower boundaries than D2 surgery in most patients. This result was concluded from the results of the Inter group 0116 study[104]. Adjuvant chemotherapy is used as the gold standard treatment in East Asian countries that typically perform standard D2 surgery[105-108]. Neo-adjuvant chemotherapy (NAC) indications are limited in these countries, and this method is accepted as an experimental treatment for most curable patients. NAC is typically only administered to patients with borderline resectable gastric cancer or a poor prognosis after R0 resection, even though evidence supported by phase 3 studies is lacking.

In a randomized controlled study of a large population in Europe, perioperative chemotherapy, including epirubicin cisplatin and 5-FU (ECF), significantly increased overall survival and cancer free survival compared to the surgery group alone (HR: 0.75, 95%CI: 0.60-0.93, P = 0.009)[103]. Other prospective studies of this procedure include the FFCD 9703 study and the EORCT 40954 study, which had relatively fewer participants. Less than 250 cases were reported in both studies, and they were ended before reaching the planned sample size[109,110]. The EORCT 40954 study does not include post-operative chemotherapy; therefore it only determines the effect of NAC compared to surgery alone. Though the FFCD 9703 study had completed data on 224 patients (the planned sample size had been 250), it was statically shown that NAC is significantly more beneficial than surgery alone (HR: 0.69, 95%CI: 0.50-0.95, P = 0.02). The EORCT study was ended due to a low enrollment rate after having recorded only 114 cases. No survival advantage was shown in this study. In the MAGIC study, the ECF regimen was used; in the FFCD 9703

study, a cisplatin regimen and 5-FU (CF) were used; in the EORCT study, cisplatin, leucovorin (FLC) and 5-FU (CF) regimens were used.

Recently, much attention has been focused on linitis plastica, which has a worse prognosis than other diseases that involve extensive lymph node invasion (either large sized lymph nodes surrounding the first branch of the celiac artery or para-aortic lymph node metastasis)[111,112]. Three phase Ⅱ clinical studies have reported a 5-year survival rate of 10% for diseases with extensive lymph node invasion. Most of these diseases have been classified as unresectable, and they are treated with palliative chemotherapy in Western countries. The survival rates were reported only in the first two studies[112,113]. Another area of focus is linitis plastica, which is accepted as inoperable by some surgeons[114].

Regarding ongoing studies, a Korean study is comparing S1 monotherapy following D2 lymphadenectomy to NAC with Docetaxel, S-1 and Oxaliplatin (PRODIGY study: NCT01515748). The RESONANCE study in China (NCT01583361) is testing the effectiveness of postoperative SOX treatment after D2 lymphadenectomy in addition to NAC with S1 and Oxaliplatin[102].

HER 2 IN GASTRIC CANCER AND TARGETED TREATMENT

Trastuzumab is a monoclonal antibody that interacts with human epidermal growth factor (HER) 2 and is related to gastric carcinoma[115]. The gene amplification and protein expression of HER2 were first reported in 1986[116,117]. Herceptin (trastuzumab) blocks HER2 function, and HER2 is a treatment option fort he breast cancer patients[118]. The anti-tumor mechanism of trastuzumab is not clearly known, but mechanisms such as blocking the cycle progression of the cell and cell signaling pathways; initiating the cell mediated cytotoxicity with antibodies; induction of anti-angiogenesis effects and increasing receptor turnover by endocytosis have been reported previously. Gene amplification of HER2 using fluorescence in situ hybridization (FISH) and protein overexpression with immunohistochemistry (IHC) have reported HER2 levels of 16%-27.1% and 8.2%-54%, respectively[119]. The trastuzumab for gastric cancer (ToGA) phase Ⅲ international multicenter RCT compared the clinical effect and safety of trastuzumab with that of standard chemotherapy (capecitabine or intravenous 5-fluorouracil and cisplatin). Survival after treatment with trastuzumab was significantly longer than that with only standard chemotherapy (13.8 mo *vs* 11.1 mo, respectively, $P = 0.0046$). Additionally, comparable toxicity and improvement of the time of progression and progression free survival were observed in the trastuzumab+ standard chemotherapy group[120]. Treatment with trastuzumab is standard for the HER2 (+) patients (IHC score +3 and/or FISH-) in the USA and Japan. Trastuzumab is recommended for patients with an IHC score of 2+/positive FISH or an IHC score of 3+ with high HER2 protein expression, according to the ToGA study in Europe. Evaluation of HER2 is essential for trastuzumab treatment[120]. The effect of trastuzumab on patients with low HER2 expression (IHC score 0/FISH positive or IHC score 1/FISH positive) is not clear according to the ToGA study. Interestingly, HER2 expression was higher in patients with gastroesophageal cancers than in those with other gastric cancers in this study (33.2% *vs* 20.9%, respectively, $P < 0.001$)[121].

In a observational, prospective, cohort, multicenter, study by Matsusaka *et al*[122], HER 2 expression and gene amplification were assessed, and the relationship between HER2 status and clinicopathological findings in Japanese gastric cancer patients with metastasis or recurrence was investigated. A total of 1461 patients in 157 centers were included in the study, and 1427 of 1461 patients were evaluated. The overall HER2 (+) patient rate was 21.2%. The rate of patients with high levels of HER2(+) (IHC score of 2+/FISH positive or IHC score of 3+) was 15.6%, and the rate of patients with low HER2 (+) levels was 7.0%. Multiple logistic regression analysis showed that an intestinal type of cancer, the absence of peritoneal metastasis and hepatic metastases are significant independent factors associated with the expression of HER2 positivity. An intestinal cancer type was associated with low HER2 expression. Factors such as the type specimen fixation, total fixation time, pH of the fixative and the time before the fixation affected the HER2 status according to this study. Additionally, the authors reported that HER2 has intratumoral heterogeneity and this rate is up to %70 in the HER2 (+) cancers. Because of that gastric biopsies can cause false negative or false positive results[122]. Therefore, endoscopists should consider conducting multiple biopsies. As a result, the intestinal type of gastric cancer is an independent factor for HER2 positivity and low HER2 expression.

The association between the HER2 gene amplification and protein expression and the clinicopathological findings of resectable gastric cancer patients were investigated in another study by He *et al*[119] A total of 197 patients who underwent curative resection were included in the study, and the survival rates were noted. The amount of HER2 gene amplification was 17.7% according to Hoffman's gastric cancer HER2 scoring system. Additionally, the HER2 (3+), HER2 (2+) and HER2 (0/1+) rates in all patients were 9.64%, 12.69% and 77.66%, respectively. The positivity of HER2 was higher in the intestinal type of cancer and well differentiated cancers than in the diffuse type and poorly differentiated cancers (28.57% *vs* 13.43%, $P = 0.0103$ and 37.25% *vs* 11.64%, $P < 0.0001$). The authors reported that gastric cancers that were well differentiated, of the intestinal type, and poorly differentiated with no metastasis to the lymph nodes

were suitable for the targeted therapy with Herceptin.

An ongoing RCT is examining the effect of trastuzumab on the HER2(+) gastric cancer patients who have undergone an extended lymphadenectomy. The results of this study will provide detailed information[123].

An accurate and standardized scoring system of HER2 is important for the Herceptin therapy and useful for the selection of gastric cancer patients.

HELICOBACTER PYLORI IN GASTRIC CANCER

Helicobacter pylori (*H. pylori*) which is involved in 90% of all gastric malignancies, infects nearly 50% of the world's population and it is the most crucial etiologic agent for gastric adenocarcinoma[124-126]. *H. pylori* infection causes some clinical manifestations such as; chronic gastritis, duodenal ulcer, gastric ulcer/adenocarcinoma and gastric mucosa-associated lymphoid tissue lymphoma (MALToma). The most important *H. pylori* releated predisposed factors for gastric carcinoma are bacterial virulence factors [cagA (cytotoxin-associated gene A) and its pathogenicity island (cag PAI) and vacA (vacuolating cytotoxin A)], host genetic factors (IL-1 gene cluster polymorphism, TNF-α and IL-10 gene polymorphism) and environmental factors (salt, smoking)[127]. *H. pylori* eradication can prevent the recurrence of peptic ulcers and MALToma of the stomach. Also recurrence rates after endoscopic resection of early gastric cancer is lower after *H. pylori* eradication. However, it is not clear that the eradication of *H. pylori* reduces the risk of gastric cancer directly. A randomized controlled trial concluded that the eradication of *H. Pylori* provided decline of gastric cancer risk significantly after 15 years of follow-up[128]. The well-known indications for *H. pylori* eradication are peptic ulcer, MALToma, and endoscopic treatment of early gastric cancer. However, Japanese guidelines strongly recommend that all *H. pylori* infections should be eradicated regardless of the associated disease[129].

The eradication of *H. pylori* varies by region. Recent Korean and Japanese guidelines still recommend Standard triple therapy (PPI + amoxicillin + clarithromycin or PPI + metronidazole + clarithromycin) as a first-line treatment[129-131]. However, recent European guidelines recommend that first-line treatment should be adjusted to clarithromycin resistance[132]. Standard triple therapy is recommended as a first-line treatment for the low-resistance (< 20%) regions, but bismuth quadruple therapy or sequential/concomitant therapy is recommended for the high-resistance (> 20%) regions[132].

Some authors suggested that, the process of *H. pylori*-related carcinogenesis is being inhibited by aspirin, NSAIDs, and COX-2 inhibitors and these can prevent the development of gastric cancer[133]. Vitamin C and antioxidants have also protective effects against *H. pylori*-induced gastric carcinogenesis[134]. In a recent meta-analysis which is including 45 randomized controlled trials, increased *H. pylori* eradication was associated with using of probiotics with standard triple therapy[135].

On the other hand, preoperative *H. pylori* infection is associated with increased survival after resection of gastric adenocarcinoma. In a multicenter retrospective study, *H. pylori* positivity was associated with longer overall survival (84.3 mo *vs* 44.2 mo, P = 0.008) for the 559 patients who had gastrectomy because of gastric cancer. *H. pylori* was not associated with recurrence free survival or disease spesific survival in all patients. Also, *H. pylori* infection showed no association with overall survival in stage 1 or stage 2 patients. But in the stage 3 patients, *H. pylori* was associated with longer overall survival (44.5 mo *vs* 24.7 mo, P = 0.018), longer recurrence free survival (31.4 mo *vs* 21.6 mo, P = 0.232), and longer disease spesific survival (44.8 mo *vs* 27.2 mo, P = 0.034)[136].

CONCLUSION

Gastric cancers are distinguished from other cancers by their high morbidity and mortality. Many studies have been conducted to improve the quality of life and extend the survival rates of patients, and some of these studies are ongoing. Although promising developments have been made in recent years, the obtained results have limited reliability and benefits. We believe that significant improvements in the treatment of gastric cancer will be developed according to the long-term results of ongoing randomized clinical trials.

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ORIGINAL ARTICLE

Basic Study

Growth hormone abolishes the negative effects of everolimus on intestinal wound healing

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Author contributions: Küper MA, Königsrainer A and Beckert S concepted the study; Küper MA, Trütschel S and Weinreich J performed the animal studies and did the laboratory work; Küper MA, Trütschel S and Beckert S interpreted the results and did literature research; all authors wrote and revised this manuscript.

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Abstract

AIM: To investigate whether the simultaneous treatment with human growth hormone (hGH) abolishes the negative effects of everolimus on anastomotic healing.

METHODS: Forty-eight male Sprague-Dawley-rats were randomized to three groups of 16 animals each (Ⅰ: vehicle; Ⅱ: everolimus 3 mg/kg po; Ⅲ: everolimus 3 mg/kg po + hGH 2.5 mg/kg sc). Animals were pretreated with hGH and/or everolimus daily for seven days. Then a standard anastomosis was created in the descending colon and treatment was continued for another seven days. The anastomosis was resected in toto and the bursting pressure was assessed as a mechanical parameter of intestinal healing. Moreover, biochemical (Hydroxyproline, PCNA, MPO, MMP-2 and MMP-9) and histological (cell density, angiogenesis, amount of granulation tissue) parameters of intestinal healing were assessed.

RESULTS: Anastomotic bursting pressure was significantly reduced by everolimus and a simultaneous treatment with hGH resulted in considerably higher values (Ⅰ: 134 ± 19 mmHg, Ⅱ: 85 ± 25 mmHg, Ⅲ: 114 ± 25 mmHg; $P < 0.05$, Ⅰ *vs* Ⅱ; $P = 0.09$, Ⅰ *vs* Ⅲ and Ⅱ *vs* Ⅲ) Hydroxyproline concentration was significantly increased by hGH compared to everolimus alone (Ⅰ: 14.9 ± 2.5 μg/mg, Ⅱ: 8.9 ± 3.6 μg/mg, Ⅲ: 11.9 ± 2.8 μg/mg; $P < 0.05$, Ⅰ *vs* Ⅱ/Ⅲ and Ⅱ *vs* Ⅲ). The number of MPO-positive cells was reduced significantly by hGH

compared to everolimus alone (Ⅰ: 10 ± 1 n/mm², Ⅱ: 15 ± 3 n/mm², Ⅲ: 9 ± 2 n/mm²; $P < 0.05$, Ⅰ *vs* Ⅱ and Ⅱ *vs* Ⅲ), while the number of PCNA-positive cells were increased by hGH (Ⅰ: 28 ± 3 /mm², Ⅱ: 12 ± 3 /mm², Ⅲ: 26 ± 12 /mm²; $P < 0.05$, Ⅰ *vs* Ⅱ and Ⅱ *vs* Ⅲ). Corresponding to these biochemical findings, HE-histology revealed significantly increased amount of granulation tissue in hGH-treated animals.

CONCLUSION: Inhibition of intestinal wound healing by everolimus is partially neutralized by simultaeous treatment with hGH. Both inflammation as well as collagen deposition is influenced by hGH.

Key words: Wound healing; Everolimus; Human growth hormone; Immunosuppression; mTOR-inhibitor; Growth hormone; Anastomotic healing

Core tip: Patients undergoing transplantation are set onto immunosuppressive medication afterwards. One agent is everolimus out of the group of the mTOR-inhibitors. Everolimus has been shown to inhibit healing of intestinal anastomoses by influencing the inflammatory phase of wound healing. Human growth hormone (hGH) has been shown to improve wound healing by increasing the amount of collagen in the wound. In this animal study we could demonstrate for the first time that a combined perioperative treatment with everolimus and hGH results in improved intestinal wound healing compared with everolimus alone. These results might be a step towards safer immunosuppression in transplanted patients.

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INTRODUCTION

Wound healing is a complex mechanism which is essential for tissue regeneration. The wound healing cascade includes three main phases, which partly overlap[1-5]. First of all there is the inflammatory phase, which lasts the first few days after wounding. In this phase, neutrophils and macrophages are attracted to the wound and produce among others proinflammatory cytokines and growth factors[6]. Thereafter, angiogenesis starts[7-11] and new collagen fibres are synthesized in the wounded area as main components of the granulation tissue representing the proliferation phase[12-17]. Simultaneously, old collagen fibres are degraded by collagenases namely the matrix metalloproteinases 2 and 9 (MMP-2/-9). Finally, there is the phase of remodeling and maturation of the formatted scar. As the proliferation phase is crucial for the stability of the wounds, impairment of the inflammatory or proliferation phases may result in severe conditions as anastomotic insufficiency.

The mammalian target of rapamycin (mTOR) is a protein kinase that regulates among others protein synthesis and cell proliferation. Its effects are mediated *via* two different complexes, the mTOR complex 1 (mTOR1) and the mTOR complex 2 (mTOR2). There are inhibitors of mTOR namely sirolimus and its derivate everolimus. *Via* inhibition of protein synthesis and cell proliferation they act as potent immunosuppressive drugs and are widely used both after bone marrow and solid organ transplantation to prevent graft rejection. However, impairment of wound healing is one major side effect of immunosuppressive drugs like tacrolimus, sirolimus or steroids[18-30]. Especially the mTOR-inhibitors have been shown to impair not only cutaneous wound healing but also have significant effects on the healing of enteral anastomoses[20,21,25-27]. These effects limit the use of the mTOR-inhibitors in the immediate postoperative period after solid organ transplantation as well as they force surgeons to switch immunosuppressive medication from mTOR-inhibitors to another, mTOR-inhibitor-free regimen prior to elective surgery. Recently, it has been demonstrated that the negative effects of everolimus on intestinal wound healing are characterized by a prolongation of the inflammatory phase of the wound healing cascade[31]. This prolongation leads to a delayed initiation of the proliferation phase of the wound healing cascade resulting in a decreased mechanical stability of the anastomoses.

On the other hand, human Growth Hormone (hGH) is an anabolic hormone produced in the pituitary gland. It has been shown previously that hGH improves intestinal wound healing[32-37]. Administration of hGH results in a 30% increase of wound collagen as well as an improved generation of granulation tissue in intestinal wounds[35]. These biochemical findings result in an enhanced intestinal wound healing under medication with hGH[32].

Aim of this animal study was to investigate whether simultaneous administration of hGH and the mTOR-inhibitor everolimus results in an improved intestinal wound healing.

MATERIALS AND METHODS

Animals

Fourty-eight male Sprague-Dawley rats (300-380 g; Harlan Winkelmann, Borchem, Germany) were kept under controlled conditions with constant temperature at 23 ℃, constant humidity of 50% and a 12 h light/12

h dark cycle. The animals were acclimatized to the laboratory conditions for at least to weeks prior to the beginning of the experiments. Water and standard rodent laboratory chow (Provimi Kliba SA, Kaiseraugst, Switzerland) were supplied ad libitum. The institutional guidelines of the University of Tübingen for care and use of laboratory animals were followed throughout the study.

Surgical procedure

Rats were anesthetized by an intraperitoneal injection of ketamine (Ketanest, 100 mg/kg; Curamed Pharma, Karlsruhe, Germany) and xylazine (Rompun, 15 mg/kg; Bayer, Leverkusen, Germany). A midline laparotomy was performed, and the descending colon was divided under protection of the mesenteric vessels. Continuity was restored with an end-to-end-anastomosis by using 10 all-layer single-stitch sutures (6/0-Prolene; Ethicon, Norderstedt, Germany). The abdomen was closed in two layers by running sutures (3/0-Ethilon; Ethicon). Postoperatively, all animals had free access to water and chow. All operations were performed by the same surgeon. Postoperative analgesia with carprofen sc twice daily (Rimadyl, 5 mg/kg; Pfizer Animal Health, New York, NY) was administered until the third postoperative day.

Study design

The study protocol was designed to minimize pain or discomfort to the animals. Animals were randomized in three groups with 16 animals each. Group Ⅰ received placebo, group Ⅱ received everolimus (RAD001; Novartis Pharma, Basel, Switzerland) in a dosage of 3.0 mg/kg alone and group Ⅲ received everolimus (3.0 mg/kg) plus human Growth Hormone (hGH, Somatropin, Novo Nordisk Pharma, Mainz, Germany) in a dosage of 2.5 mg/kg. Everolimus was administered daily by gastric gavage, while hGH was administered daily subcutaneously. The first dose was given 7 d prior to surgery, and the medication was continued over a period of 14 d. At day 7 after surgery, rats were sacrificed by intracardiac puncture under isoflurane anesthesia. Relaparotomy was performed and the anastomotic region was resected over a length of 40 mm with the suture line in the middle. Surrounding tissues or adhesions were resected with the anastomosis. The following parameters were assessed in the anastomotic region: anastomotic bursting pressure, histology [hematoxylin and eosin (HE) and Azan staining], quantification of hydroxyproline, immunohistochemical staining for proliferating cell nuclear antigen (PCNA) and for myeloperoxidase (MPO), and zymography for quantification of the matrixmetalloproteinases MMP-2 and MMP-9.

Anastomotic bursting pressure

The resected colon segment with the anastomosis in the middle was cleared carefully of mesenteric fat. Feces were removed, and the segment was washed gently in sterile saline. One end of the bowel was connected to an infusion pump; the other end was connected to a manometer, which registered the increasing intraluminal pressure graphically and numerically. The bowel lumen was then infused with sterile isotonic saline solution at an infusion rate of 1 mL/h. The bursting pressure was defined as the highest pressure that was resisted by the bowel segment[38].

Quantification of hydroxyproline

After the assessment of bursting pressure, the anastomotic segment was bisected longitudinally into two-third and one-third segments as previously described by Agren *et al*[39]. Punch biopsies (4 mm diameter) were taken from the sutured area of the two-third longitudinal segment and stored in liquid nitrogen or dried for 48 h at 37 ℃. Tissue dry weight (DW) was measured, and the content of hydroxyproline was then determined by the method of Woessner, modified by Stegemann and Stalder[40].

Histology and immunohistochemistry

For the histopathologic and immunohistochemical analyses, the one-third longitudinal segment was pinned to a cork plate and immersed into 4% paraformaldehyde overnight at 4 ℃, dehydrated in alcohol, and embedded in paraffin. Tissue was cut in 1mm serial cross sections, deparaffinized, and stained with H and E. Cell proliferation was assessed by proliferating cell nuclear antigen antibody (PCNA, Oncogene Science, Uniondale, NY) and inflammatory activity of neutrophil granulocytes by myeloperoxidase antibody (MPO, Dianova, Germany). These assays use the avidin-biotin complex method with 3,3′ diaminobenzidine serving as chromagen. Tissue sections were incubated overnight at 4 ℃ with PCNA or MPO antibody (1:75) followed by incubation with biotinylated secondary antibody as previously described[41].

The analysis of PCNA and MPO positive cells was performed in the granulation tissue of the anastomosis. In total, four high power fields (× 400) of granulation tissue (two at each margin) were examined in every anastomosis. Stainings were evaluated by positive stained cells per area using the Quantimet system (Leica, Jena, Germany). Anastomotic morphology was assessed in H and E-stained slides by standard light microscopy. Analysis was performed semiquantitatively (grade 0-3 for each parameter) under a binocular light microscope (× 400). Histologic parameters were defined as cell density and the amount of granulation tissue in the anastomotic region. Angiogenesis was quantified by the extent of vessel growth at the anastomotic site. Samples were analyzed by an independent blinded investigator.

For the visualization of the collagen fibers,

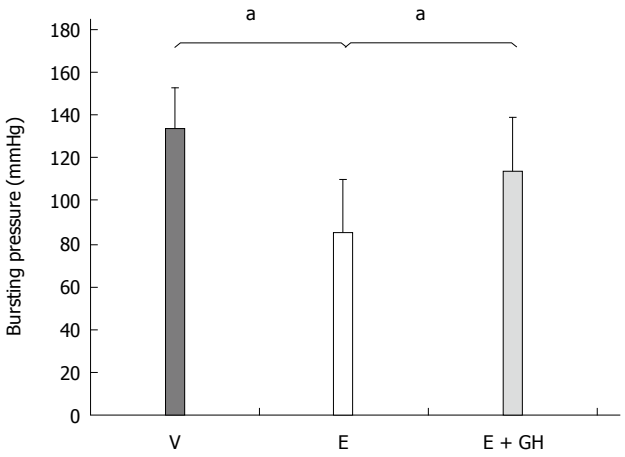

Figure 1 Bursting pressure of the colonic anastomosis (mmHg). Everolimus treatment decreased the bursting pressure significantly. Additional treatment with human growth hormone (hGH) partial antagonized this effect. V: Vehicle, E: Everolimus 3.0 mg/kg, E + GH: Everolimus 3.0 mg/kg + hGH 2.5 mg/kg; [a]*P* < 0.05, V *vs* E, E *vs* E + GH.

samples from the anastomosis were stained with the Azan method[42]. These samples were also analyzed semiquantitatively by an independent blinded investigator.

Zymography

Zymography was performed with equal amounts of pooled anastomotic protein samples from all animals of each group. The presence of MMP-2 and -9 activities was demonstrated by the use of 10% SDS-PAGE containing 10% gelatine (Ready Gel Zymogram; Bio-Rad Laboratories GmbH, Munich, Germany) under nonreducing conditions. Gels were washed twice with 2.5% Triton X-100 (Sigma-Aldrich, Seelze, Germany), incubated for 48 h at 37 ℃ in incubation buffer (50 mmol/L TRIS pH 7.0, 5 mmol/L $CaCl_2$, 200 mmol/L NaCl, 1 mmol/L $ZnCl_2$, 0.05% Brij35, 0.05% NaN_3), and stained with 0.1% Coomassie brilliant blue. Proteolytic activities were visualized by clear zones indicating the lysis of gelatine. The lytic zones on the zymograms were defined as MMP-2 or MMP-9 according to the size standard.

Statistical analysis

Data are expressed as mean ± SD unless otherwise stated. Differences between the groups were calculated by the Mann-Whitney-*U* test. For multiple comparisons, values were adjusted according to Bonferroni. A *P*-value < 0.05 was considered significant.

The statistical methods of this study were reviewed by the Institute of clinical epidemiology and applied biostatistics from the University of Tübingen.

RESULTS

All animals survived the procedure and no anastomotic dehiscence or peritonitis were found during re-laparotomy.

Bursting pressure

The anastomotic bursting pressure was significantly reduced by everolimus (Ⅰ: 134 ± 19 mmHg, Ⅱ: 85 ± 25 mmHg, *P* < 0.05) (Figure 1). Simultaneous treatment with hGH resulted in a higher bursting pressure (Ⅲ: 114 ± 25 mmHg). However, this increase was not statistically significantly both compared to groups Ⅰ and Ⅱ (*P* = 0.09, Ⅲ *vs* Ⅰ and Ⅲ *vs* Ⅱ each).

Histology

In the H and E stained samples there were significant changes in anastomotical architecture in everolimus-treated animals, which were reduced by simultaneous treatment with hGH (Figure 2A-C). Azan staining revealed a decreased arrangement of the collagen fibres under treatment with only everolimus compared to placebo and simultaneous treatment with hGH (Figure 3A-C).

Anastomotic hydroxyproline content

Everolimus significantly reduced the hydroxyproline content in the anastomotic region, while simultaneous treatment with hGH inverted this negative effect completely (Ⅰ: 14.9 ± 2.5 μg/mg DW, Ⅱ: 8.9 ± 3.6 μg/mg DW, Ⅲ: 11.9 ± 2.8 μg/mg DW, *P* < 0.05, Ⅰ *vs* Ⅱ and Ⅰ *vs* Ⅲ and Ⅱ *vs* Ⅲ; Figure 3D).

PCNA and MPO immunohistochemistry

Everolimus decreased PCNA expression significantly compared to placebo treatment (Ⅰ: 28 ± 3 n/mm², Ⅱ: 12 ± 3 n/mm², *P* < 0.05), while simultaneous treatment with hGH antagonized this effect completely (Ⅲ: 26 ± 12 n/mm², *P* < 0.05 Ⅱ *vs* Ⅰ and Ⅲ; Figure 4A). On the other hand hGH lead to normalization of the number of MPO-positive cells compared to only-everolimus-treated animals (Ⅰ: 10 ± 1 n/mm², Ⅱ: 15 ± 3 n/mm², Ⅲ: 9 ± 2 n/mm²; *P* < 0.05, Ⅱ *vs* Ⅰ and Ⅲ; Figure 4B).

Zymography

Everolimus-treated animals had significantly increased activity-levels of the pro-inflammatory zytokines MMP-2 and MMP-9 in the anastomotic region. Simultaneous treatment with hGH reduced these activity-levels to nearly-normal values (Figure 5A-C).

DISCUSSION

Wound healing is a complex biological process which is essential for healing of injured tissue. There are different phases of wound healing finally leading to a stable scar in the wounded tissue. After initial cleaning of the wound by macrophages and neutrophil granulocytes the white cell population shifts to predominantly macrophages, which consume large amounts of oxygen by their respiratory burst which produce proinflammatory cytokines, enzymes (*e.g.*, the matrix-metalloproteinases MMP-2 and MMP-9) and growth factors to remove destroyed collagen from the wounded tissue and to attract cells to the wound

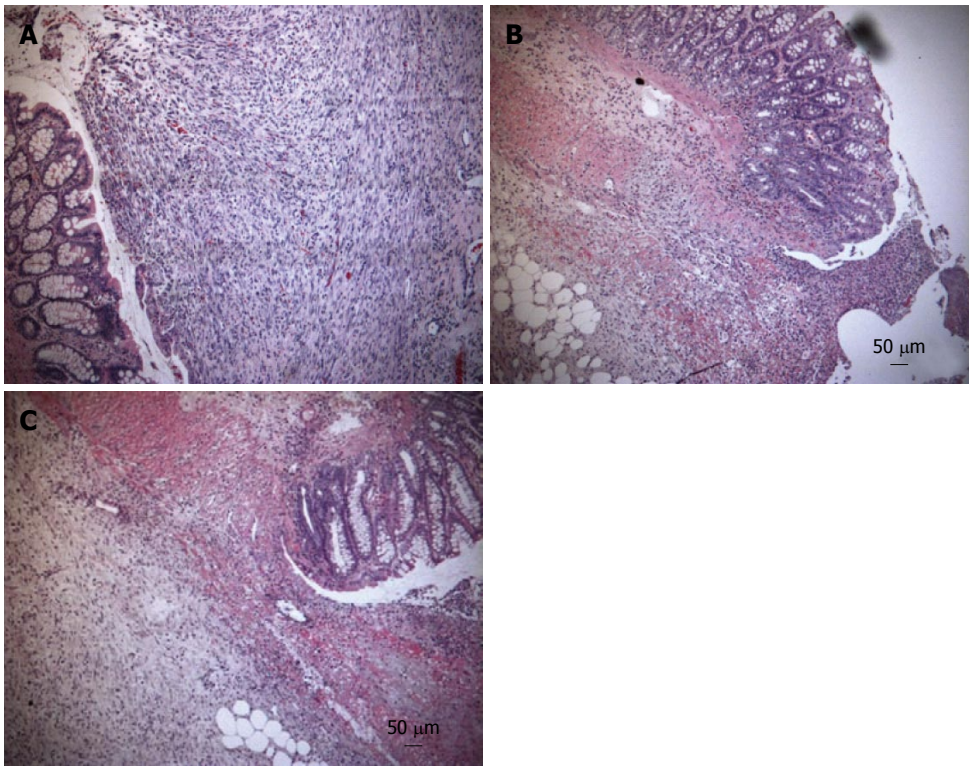

Figure 2 Hematoxylin and eosin staining from the anastomotic region. In the hematoxylin and eosin stained samples there were significant changes in anastomotical architecture in everolimus-treated animals, indicated by decreased cell density and reduced angiogenesis (B) which are indicators for granulation tissue. Simultaneous treatment with human growth hormone (hGH) showed more granulation tissue (C). A: Vehicle; B: Everolimus 3.0 mg/kg; C: Everolimus 3.0 mg/kg + hGH 2.5 mg/kg (magnification × 100).

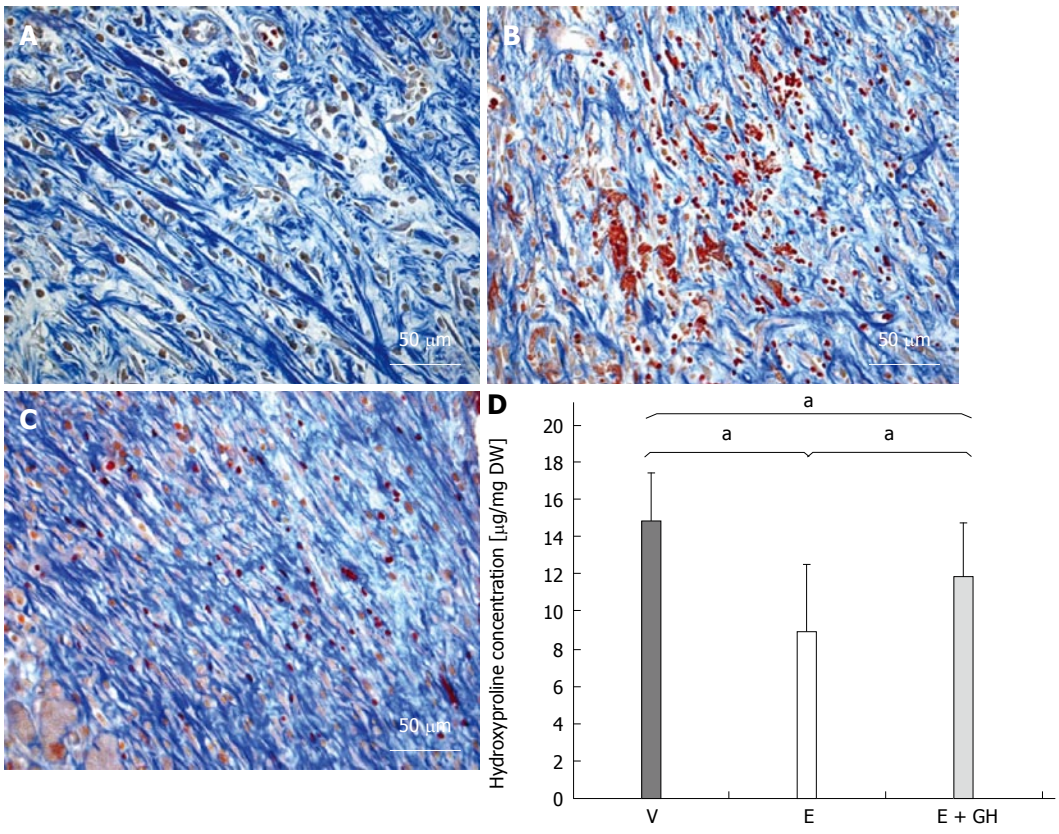

Figure 3 Azan staining from the anastomotic region. The collagen fibres appear blue. Everolimus treated animals show a disturbed arrangement of collagen fibres, which is antagonized by additional human growth hormone (hGH)-treatment (A-C). A: Vehicle; B: Everolimus 3.0 mg/kg; C: Everolimus 3.0 mg/kg + hGH 2.5 mg/kg. Hydroxyproline concentration in the anastomotic region in mg per mg dry weight (DW). The decrease of hydroxyproline under everolimus-treatment is reduced by additional hGH-treatment (D) (magnification × 400). V: Vehicle, E: Everolimus 3.0 mg/kg, E + GH: Everolimus 3.0 mg/kg + hGH 2.5 mg/kg; $^{a}P < 0.05$, *vs* E group, V *vs* E + GH group.

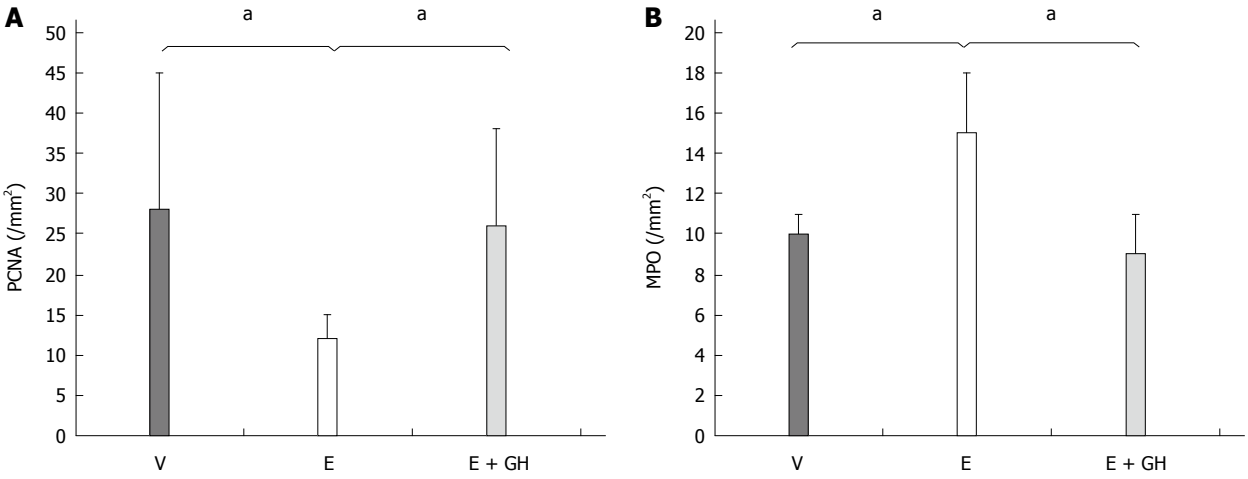

Figure 4 Immunohistochemistry for PCNA- (A) and MPO- (B) positive cells from the anastomotic region. Treatment with everolimus alone reduced significantly the amount of PCNA-positive cells in the anastomotic region while increasing the number of MPO-positive cells, indicating a prolonged inflammation. Simultaneous treatment with hGH led to nearly normal values for PCNA- and MPO-positive cells**.** V: Vehicle, E: Everolimus 3.0 mg/kg, E + GH: Everolimus 3.0 mg/kg + hGH 2.5 mg/kg; V *vs* E, E + GH *vs* E, $^{a}P < 0.05$.

which are essential for the proliferation phase[3,43]. This particular step of the healing cascade is defined as the so-called inflammatory phase; its activity peaks in the first 3 to 5 d after wounding[2,44]. Subsequently, healing progresses to the phases of proliferation and tissue remodeling, which are mainly characterized by collagen synthesis by the fibroblasts and new vessel growth to provide enough oxygen to the wound[3,44]. In this phase fibroblasts produce mainly collagen to repair the wound. Moreover, angiogenesis is started to provide enough oxygen to the wound. If these two phases of wound healing are disturbed, it may result in severe wound healing problems which can be devastating in cases of non-healing anastomoses in the gastrointestinal tract. mTOR is a key factor in the regulation of protein synthesis and cell proliferation. Its effects are mediated *via* complex building with two complexes being involved [mTOR-complex 1 (mTORC1) and mTOR-complex 2 (mTORC2)]. mTORC1 is essential in the regulation of protein synthesis, while the main function of mTORC2 is regulation of the actin cytosceleton. Both, mTORC1 and mTORC2 are activated amongst others by insulin-like growth factor 1 (IGF-1), a growth factor, which is stimulated by the human growth hormone (hGH). IGF-1 acts *via* the Protein-Kinase B (PKB), also known as Akt, and the corresponding Akt-pathway, resulting in complex-building of mTORC1 and thus activation of protein synthesis. This has been demonstrated in animal studies previously. Christensen *et al*[34] investigated the effects of hGH on the healing of rodent colon anastomoses in the 1990s[35,45-49]. They found an increased bursting pressure in the hGH-treated animals which showed a significantly increased amount of collagen in the anastomotic region compared to the control groups[35,45,46]. Beckert *et al*[32] demonstrated in another animal study that administration of hGH leads to increased healing of gastric ulcers in rats compared to the control groups. They showed an increased cell proliferation and angogenesis, both key factors of wound healing[3,44].

On the other hand, mTOR-inhibitors have been demonstrated to significantly disturbe wound healing. Initially there were clinical reports of patients after kidney transplantation who received sirolimus as immunosuppressant drug. These patients had significantly more wound complications than patients with a mTOR-inhibitor-free immunosuppressant regime[21,22,50]. The same was demonstrated later on after liver transplantation[19,20]. van der Vliet *et al*[27] then showed in 2011 that the hydroxyproline-content in healing colon anastomoses in rats was significantly decreased after treatment with everolimus. With hydroxyproline acting as a progenitor of collagen, this demonstrated for the first time, that mTOR-inihibtors result in a decreased wound strength by reducing protein synthesis. A similar way of action was shown in 2011, when it was demonstrated in the same experimental setting like in this study, that everolimus resulted in a decreased hydroxyproline-content in colonic anastomotic tissue. Furthermore, cell proliferation was reduced as well as angiogenesis while the inflammatory reaction in the anastomosis was increased by means of increased myeloperoxidase (MPO)-positive cells and increased activity of the matrix-metalloproteinases 2 and 9 (MMP2/9) indicating a delayed phase of proliferation[31].

The combination of hGH and everolimus resulted in an improved wound healing compared to everolimus alone. Bursting pressure, content and organization of collagen in the anastomotic tissue, as well as histological parameters like cell density and angiogenesis were significantly improved after combination treatment with hGH and everolimus compared to everolimus alone. All of these factors contribute the proliferation phase of wound healing and were increased, whilst

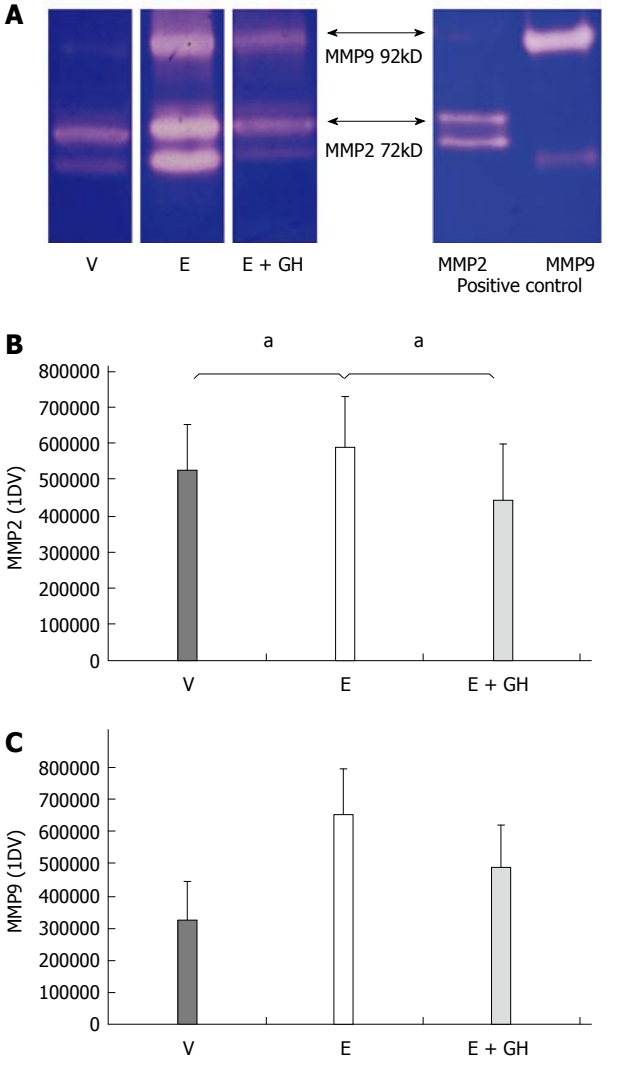

Figure 5 Zymographic activities of the pro-inflammatory matrix-metalloproteinases MMP2 and MMP9. Zymography gel showing increased proteolytic activity under treatment with everolimus which is reduced by simultaneous treatment with human growth hormone (hGH) (A). Numeric calculation of the proteolytic activity of MMP2 (B) and MMP9 (C). V: vehicle, E: everolimus 3.0 mg/kg, E+GH: everolimus 3.0 mg/kg + hGH 2.5 mg/kg; V *vs* E, E + GH *vs* E, $^{a}P < 0.05$.

MPO-positive cells and activity of MMP-2 and -9, both markers of the inflammatory phase of wound healing, were reduced by combination treatment of hGH and everolimus. This indicates a partial normalization of wound healing. However, all of these factors were still reduced compared to placebo treatment. One possible explanation is the activation of the Akt-pathway by an IGF-1 synthesis, which is stimulated by hGH. This Akt-pathway leads to an increased forming of the mTOR-complex 1 (mTORC1). On the other hand, everolimus binds to this mTORC1 with high affinity resulting in reduction of the mTORC1-mediated protein-synthesis and cell proliferation. With increased amounts of mTORC1 due to the hGH-treatment, higher doses of everolimus should be needed to produce the same effects as without simultaneous hGH-treatment. However, this has to be investigated in further studies.

In conclusion we could demonstrate for the first time that simultaneous treatment of everolimus and hGH can ameliorate the negative effects of everolimus on intestinal wound healing. However, the exact mechanisms of this partial antagonization remain unclear by now and have to be further investigated.

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COMMENTS

Background

Mammalian target of rapamycin (mTOR)-inhibitors are part of standard immunosuppressive medication after both solid organ and bone marrow or stem cell transplantation. The mostly used agents are sirolimus and its derivate everolimus. However, due to severe impairment of wound healing, the definitive mTOR-based immunosuppressive regime is often initiated weeks after transplantation. Moreover, in case of some medical interventions such as surgical procedures the regime has to been switched to an mTOR-inhibitor-free regime with an increased risk of adverse events like graft rejection. To reduce this risk we need to better understand the mechanisms of mTOR-inhibitor-induced impairment of wound healing. One aspect is the delayed inflammatory phase of wound healing resulting in a decreased deposition of wound collagen. So there might be a point of application to reduce this risk.

Research frontiers

The delayed inflammatory phase of wound healing probably is only one mechanism of action of impaired wound healing induced by mTOR-inhibitors as the exact working mechanisms are not known in detail yet. Moreover it is not known yet whether the immunosuppressive potential of the mTOR-inhibitors is caused by the same pathway.

Innovations and breakthroughs

This is the first study which evaluated an approach to counter the negative effects of everolimus on the wound healing and the first time that it was demonstrated that the negative effects of everolimus on wound healing can be antagonized at least partly by another agent.

Applications

The present results might be used to better understand the mechanisms of action of mTOR-inhibitors as well as the basis for further investigation of wound healing under mTOR-inhibitor medication. In the end there is the goal that changing of mTOR-inhibitor-based immunosuppressive regimes is not necessary any more in advance of a planned medical intervention or after an emergency surgical procedure.

Peer-review

The research topic has significant application for immunosuppressant medications in patients undergoing solid organ transplantation

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ORIGINAL ARTICLE

Basic Study

Effect of the manipulation of the duodenal papilla during double balloon enteroscopy

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Abstract

AIM: To determine the hypothesis that inflating the balloons in the duodenal papilla determines changes in the biochemical markers of pancreatitis.

METHODS: Four groups of pigs were used: Group papilla (GP), the overtube's balloon was inflated in the area of the papilla; GP + double balloon enteroscopy (GP + DBE), the overtube's balloon was kept inflated in the area of the papilla for 20 min before a DBE; Group DBE (GDBE), DBE was carried out after insuring the balloon's inflation far from the pancreatic papilla; and Group control (GC). Serum concentrations of amylase, lipase and C-reactive protein (CRP) were evaluated. Pancreases were processed for histopathology examination.

RESULTS: Main changes occurred 24 h after the procedure compared with baseline levels. Amylase levels increased significantly in GP (59.2% higher) and were moderately higher in groups GP + DBE and GDBE (22.7% and 20%, respectively). Lipase increased in GP and GP + DBE, whereas it hardly changed in GDBE

and in GC. CRP increased significantly in GP, GP + DBE and GDBE, while no changes were reported for GC. No statistically significant difference between groups GP and GP + DBE was found for the histopathological findings, except for vacuolization and necrosis of the pancreatic parenchyma that was higher in GP than in GP + DBE.

CONCLUSION: The manipulation of the duodenal papilla by the inflated overtube's balloon during DBE causes pancreatic structural damage and increased biochemical markers associated with pancreatitis.

Key words: Duodenal papilla; Double balloon enteroscopy; Pancreas; Animal model; Pancreatitis

Core tip: During double balloon enteroscopy (DBE) the manipulation of the duodenal papilla by the inflated balloons around the area of secretion of the pancreas determines structural damage in the organ and increased levels of biochemical markers of pancreatitis. Thus, the widely assumed recommendation of avoiding any contact of the balloons with the duodenal papilla so as to decrease post-DBE pancreatic risk is now supported by empirical results in an animal model.

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INTRODUCTION

Double balloon enteroscopy (DBE) is a variety of push and pull endoscopy which allows diagnostic and therapeutic actions deep in the small intestine[1]. Although DBE has been considered a reasonably safe technique[2], articles reviewing complications have been published recently[3]. Among the major complications (0.72%)[4] acute post-procedure pancreatitis is the most severe[5]. DBE can be done by oral or anal approaches but post-DBE pancreatitis is mainly related with the anterograde approach. The average incidence of this complication is not very high (0.3%)[6] however, it may be underestimated in previously published data[7]. With the use of the oral approach increases in the incidence of pancreatitis which may vary from 1%-3%[2,7,8] to 12.5[9].

There is some controversy about the etiology of acute pancreatitis after DBE but most opinions agree that the cause is related with the technique itself[2,10-13]. Ischemia of the pancreas from prolonged mechanical stress due to repeated stretching of the endoscope, reflux of duodenal content into the pancreatic duct consequent to overpressure in the intestinal compartment, and disturbance of the pancreatic secretion due to direct trauma to the papilla of Vater are the most plausible theories[6,7,11-13]. In order to avoid potential trauma in the ampullar zone some endoscopists recommend inflating the balloons after passing the ligament of Treitz[14,15], although no causal relationship has been found with a lower incidence of hyperamylasemia or reduction in pancreatitis rate[16]. However, precise control of this manoeuvre is not always guaranteed[2] and during the learning curve, unintentional shearing of the ampullary area is likely.

Post-DBE hyperamylasemia is the most frequent biochemical marker associated with suspicion of acute pancreatitis[10,11,13], however increased amylase levels after DBE are not always indicatory of pancreatic inflammation since asymptomatic hyperamylasemia is quite common in DBE procedures[2]. Lipase levels are more specific for the pancreatic function but post-DBE hyperlipasemia is not always accompanied by clinical signs of pancreatitis such as increased abdominal pain. Nevertheless, amylase and lipase levels together plus those of the reactive C-protein (CRP) are a valuable tool to diagnose post-DBE acute pancreatitis because they are commonly elevated soon after the procedure[2,9].

One major limitation for research in this field comes from the fact that DBE is mainly restricted to endoscopy procedures in humans, although the use of an appropriate animal model could help to overcome this limitation. The porcine model has been used both *ex vivo* and *in vivo* for DBE training and research, helping to improve the technique conditions in humans[17-19]. Pig and human pancreas are partially retroperitoneal, encircle the portal vein and have similar parenchyma firmness. Unlike humans, pigs have a unique pancreatic duct (accessory) which opens at a minor duodenal papilla located 8-10 cm distally from the major duodenal papilla and approximately 12-15 cm from the pylorus[20].

In the present work a pig model was used to test the hypothesis that the length of time the inflated balloons are kept in contact with the duodenal papilla determines changes in the pancreas structure, as well as the biochemical markers of pancreatitis. This study might help to reach a better understanding of the factors involved in the etiology of pancreatitis post-DBE.

MATERIALS AND METHODS

The experimental work was carried out at the Minimally Invasive Surgery Center Jesús Usón (CCMIJU) (http://www.ccmijesususon.com). All animals received humane care in compliance with the European Communities Council Directive (86/609/EEC) and protocols were approved by the Ethics Committee for Animal Research of the University of Murcia (Ref.452/2009). An EN-

Table 1 Evaluation of pancreatic lesions

Parameter/lesion	Grade 0	Grade 1	Grade 2	Grade 3
Edema	Absent	Diffuse dilation of interlobular septi	Diffuse dilation of interacinous space	Diffuse dilation of intercellular space
Vacuolization	Absent	Focal (< 25%)	Diffuse (25%-50%)	Severe (> 50%)
Necrosis	Absent	< 50% lobules	50%-75% lobules	> 75% lobules
Vessels congestion	Absent	Focal (< 25%)	Diffuse (25%-50%)	Severe (> 50%)
Inflammation	Absent	< 50% lobules	50%-75% lobules	> 75% lobules

450T5 enteroscope (Fuji Film) for exclusive use in animals was used in this work.

Thirty large white pigs (35-40 kg) were used in this study. Pigs were randomly assigned to one of the following groups: GP, GP + EDB and GC.

GP (Group papilla) (n = 10) aimed at evaluating the effect of shearing the minor duodenal papilla independently of DBE. In this group the overtube's balloon was inflated in the area of the papilla, kept there for 90 min and then removed without DBE exploration. GP + DBE (Group papilla + DBE) (n = 10) was designed to simulate a potential situation (beginner's scenario) where papilla compression might occur during the first manoeuvres of DBE exploration. In this group the overtube's balloon was kept inflated in the area of the papilla for 20 min before proceeding with a standard DBE exploration of 90 min. A control group GC (n = 10) for both papilla compression and DBE was used for comparison with GP, GP+DBE. In the control group a conventional esophagogastroduodenoscopy (EGD) was carried out. A forth group was considered GDBE (Group DBE), it represented the most common situation in conventional oral DBE (clinical situation). DBE was carried out for 90-140 min after insuring the balloon´s inflation occurred distal to the pancreatic papilla. The data from GDBE came from work previously published by our group[19].

Before procedure all pigs were fasted for 24 h and then intramuscularly pre-medicated with diazepam 0.1 mg/kg, ketamine 10 mg/kg and atropine 0.01 mg/kg. General anaesthesia was induced with propofol 2 mg/kg intravenously and maintained with Sevofluorane 1.8%-2% delivered *via* endotracheal tube. DBE were always performed by two of the authors (Pérez-Cuadrado E or Soria F) who have been routinely performing this technique for at least 9 years. During DBE exploration insertion depth was estimated according to the methodology established by May *et al*[17], which has also been validated in the pig model[21]. After anesthesia, recovering pigs were checked for 24 h for signs of decreased activity, irritability, vomiting or anorexia. Blood samples were taken before procedure (Basal), at the end of the procedure (End) and just before euthanasia (24 h). The serum concentrations of amylase, lipase and C-reactive protein (CRP) were evaluated. Euthanasia was performed by a pentobarbital overdose.

Immediately after death, pancreases were examined *in situ* and carefully removed from the abdominal cavity, and the left pancreatic lobe (tail) immersed in 10% buffered formaline. The samples for histopathology were systematically taken, making blocks of 1 cm^3 (8-12 blocks per pancreas). Tissues were embedded in paraffin, sectioned at 4 microns, and stained with hematoxylin and eosin. Histology sections were studied under light microscopy. The presence and distribution of lesions were evaluated for edema, vacuolization, necrosis, vascular congestion and inflammation. Histopathological findings were characterized and categorized by a pathologist (Candanosa E, author) as showed in Table 1. GDBE histopathology was not included because in this group euthanasia was performed 7 d after procedure[19].

The statistical analysis was carried out with the SPSS 19.0 (SPPS Inc) package. For the biochemical markers (amylase, lipase and CRP) descriptive statistics were calculated and the analysis of variance (ANOVA, linear model with repeated measures) performed considering the different timing of blood sampling as within-subject factors. To evaluate the significance of the histopathological findings the severity (graded 0-3) was compared between the experimental groups by the non-parametric Mann-Whitney test. In addition, the potential association between the histopathological features and the experimental groups was evaluated by contingency tables and the χ^2 test. All statistics were initially performed for a significance level of $P < 0.05$.

RESULTS

Pancreatic markers /Biochemical evaluation

The average serum concentrations of amylase, lipase and CRP at the different sampling periods (Basal, End of experiment and 24 h post-DBE) have been summarized in Table 2 (within groups comparison) and Figure 1 (between groups comparison).

Twenty four hours after the procedure the amylase levels increased quite significantly, such that compared to the basal situation it was 59.2% higher in group GP, and moderately higher in groups GP + DBE and GDBE (22.7% and 20% increases, respectively). However, the amylase levels in GC (control group) hardly changed from the basal levels throughout the experiment (Table 2).

Amylase levels ranged between a maximum in GP and minimum in GC with differences between

Table 2 Within group comparison of serum levels (mean ± SD) of biochemical markers (amylase, lipase and CRP) at different sampling points (Basal, End of endoscopy and 24 h later)

Group	Time	Amylase (IU/L)	Lipase (IU/L)	CRP (mg/L)
GP	Basal	2507.9 ± 553.6[1]	34.3 ± 19.26[1]	30.1 ± 43.3[1]
	End	2317 ± 523.5[2]	45.4 ± 33.6[1]	27.4 ± 41.7[1]
	24 h	3993.3 ± 2047.3[3]	444.6 ± 417.2[2,d]	182.3 ± 44.5[2,d]
GP + DBE	Basal	2214.9 ± 385.9[1]	19.6 ± 16.4[1]	20.6 ± 26.4[1]
	End	1920.2 ± 360.8[2]	37.1 ± 29.5[2,b]	16.2 ± 21.8[1]
	24 h	2717.2 ± 686.6[2]	146.8 ± 128.1[1,2]	117.3 ± 46.1[2,d]
GDBE	Basal	1968.1 ± 929.6[1]	17.7 ± 7.7[1]	39.1 ± 41.2[1]
	End	1850 ± 820.7[2]	8.8 ± 3.6[1]	37.2 ± 38.3[1]
	24 h	2487.2 ± 1093.4[2]	26.7 ± 20.9[1]	114.8 ± 100.7[2,d]
GC	Basal	1965.4 ± 856.4[1]	10.7 ± 3.1[1]	9.8 ± 8.8[1]
	End	1881.9 ± 839.5[1]	9.1 ± 2.32[1]	10.1 ± 8.3[1]
	24 h	2245.8 ± 995.9[1]	37.5 ± 23.6[1]	24.2 ± 17.4[1]

[1,2,3]Different superscripts within the same column of the same experimental group indicate significant differences at 95% confidence ($P < 0.05$). [b]$P < 0.01$, [d]$P < 0.0001$ *vs* basal. GP: Group papilla; GP + DBE: Group papilla + double balloon enteroscopy; GC: Group control; GDBE: Group DBE.

these two groups. Despite of the fact that the highest amylase levels were found in GP, no significant differences were found between GP, GP + DBE and GDBE at any of the three sampling points (Figure 1A).

The lipase concentration hardly changed throughout the procedure ranging between 8.8 and 45.4 IU/L. However, 24 h after the procedure lipase levels in the papilla-focused groups (GP and GP+DBE) were seen to rise sharply, while hardly changed in those groups where the papilla was not manipulated (GDBE and GC) (Figure 1B). Compared to the basal situation the lipase concentration was 11.4 and 7.5 times higher in GP and GP + DBE, respectively. Thus, inflation and maintenance of the balloons around the papillar area resulted in dramatic changes in the lipase levels one day after the procedure.

None of the procedures resulted in immediate changes in CRP levels (Table 2). The basal CRP range was 11-39.1 mg/L, which included the values observed at the end of the procedures. One day later CRP increased significantly in GP, GP + DBE and GDBE, with values of 5.5 and 6.3 and 3.6 times higher than basal levels, while no significant changes were reported in GC (Table 2).

From a clinical point of view it is important to note that none of the pigs showed significant alterations in the monitored parameters during the procedure (blood pressure, heart and respiratory rates and oxygen saturation). The pigs ate normally and showed no clinical signs of abdominal pain, vomiting, diarrhea or altered sensory behavior in the 24 h following endoscopies.

Histopathological findings

Results from the control group (GC) revealed the absence of edema, vacuolization, necrosis, vascular congestion and inflammation. However, all these pathological features were observed to a certain degree in pancreas samples from groups GP (Figure 2) and GP + EDB (Figure 3). No statistically significant differences between these two groups were found for edema, vessels congestion and inflammation, whereas vacuolization and necrosis of the pancreatic parenchyma was higher in GP than in GP + DBE (Table 3). This result was confirmed by the χ^2 test as the frequency of samples with vacuolization and necrosis was significantly different in GP and GP+DBE groups with a 90% of confidence ($P < 0.1$) (Table 4).

DISCUSSION

In this study the manipulation of the duodenal papilla by the inflated balloons during DBE was shown to cause pancreatic structural damage and concomitant increase of biochemical markers of pancreatitis. This direct association was experimentally demonstrated in a pig model using two experimental groups (GP and GP+DBE) with different time periods of contact between the balloons and the duodenal papilla. Results were conclusive, the longer the manipulation of the papilla the higher the changes in biochemical markers. Thus, the widely assumed recommendation of avoiding any contact of the balloons with the duodenal papilla so as to decrease post-DBE pancreatic risk[9,14] is now supported by empirical results in an animal model.

Contact between the duodenal papilla and the inflated balloon for 90 min resulted in a very significant increase in amylase (59.2%), lipase (11.4 times) and CRP (5.5 times) levels 24 h after endoscopy (Figure 1). This information gives a clear indication of how the manipulation of the duodenal papilla influences the pancreas independently of DBE. The combined effect of papilla manipulation and DBE was specifically monitored in GP + DBE. Although the results were less dramatic, GP + DBE procedure also resulted in significant increases of amylase (22.7%), lipase (7.5 times) and CRP (6.3 times) levels 24 h after the procedure. In contrast, in GDBE serum levels of amylase and lipase never reached twice the baseline levels (20% for amylase and 1.5 times for lipase), as

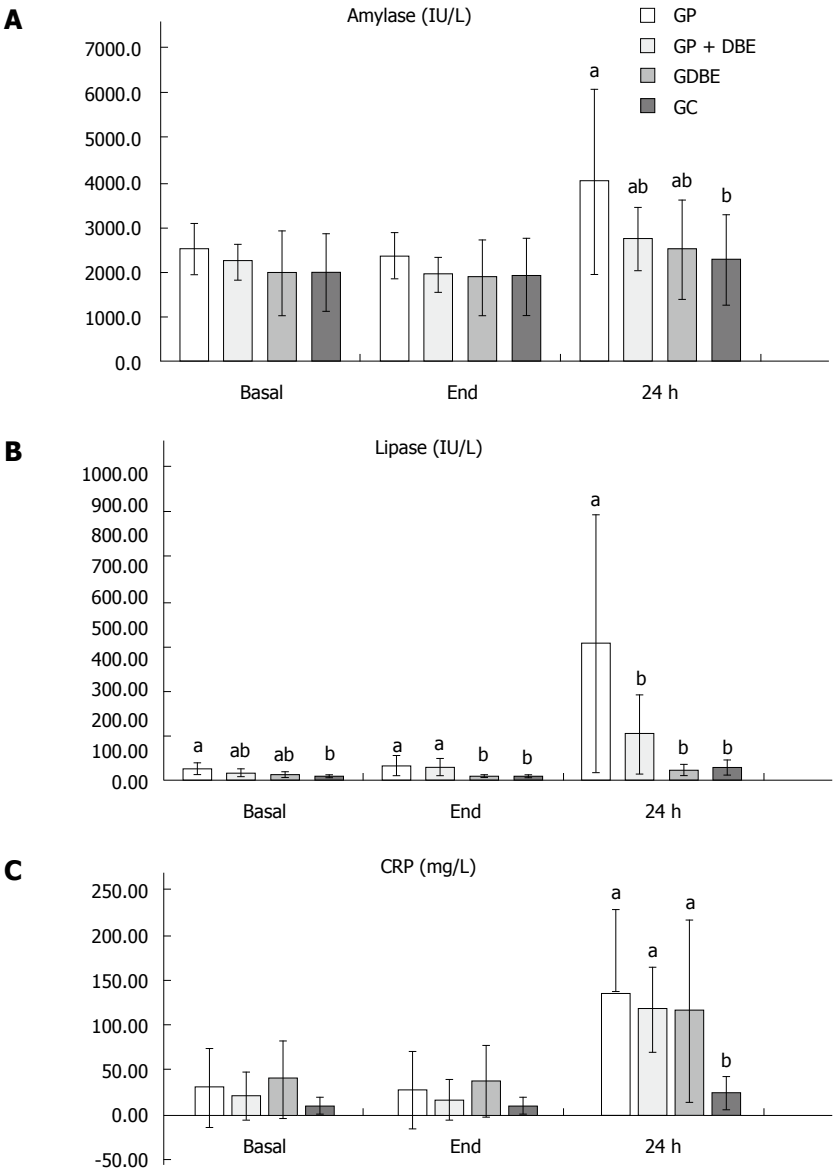

Figure 1 Between groups comparison of serum levels of amylase (A), lipase (B) and CRP (C) at different sampling times (Basal, End of endoscopy and 24 h later, mean ± SD). [a,b]Different superscripts over the bars within the same time interval indicate significant differences between GP, GP + DBE, GDBE and GC at 95% confidence ($P < 0.05$). GP: Group papilla; DBE: Double balloon enteroscopy; GC: Group control; GDBE: Group DBE.

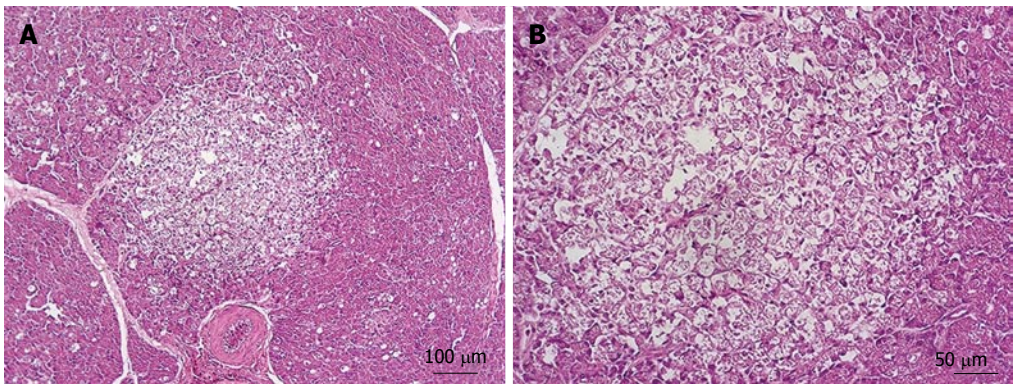

Figure 2 Photomicrographs of the left lobe (tail) of porcine pancreas group Group Papilla. A: Pancreas showing focal necrosis perivascular with evidence of vacuolization in acinar cells; B: Magnification of previous image, view of acinar cell´s cytoplasm vacuolated. The nuclei are fragmented and shrunken. Hematoxylin and eosin staining.

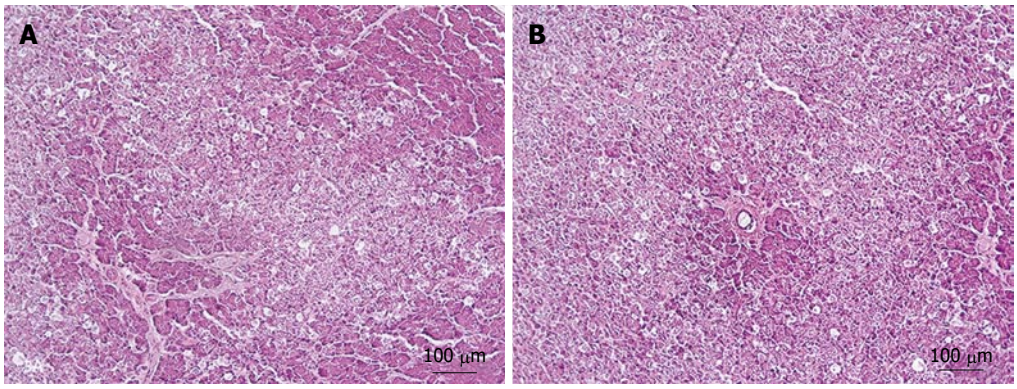

Figure 3 Photomicrographs of the left lobe (tail) of porcine pancreas of group GP + DBE. A: Pancreas with different degree of perivascular necrosis and low level of vacuolization; B: Pancreas with perivascular coagulative necrosis. Hematoxylin and eosin staining. GP: Group papilla; DBE: Double balloon enteroscopy.

Table 3 Comparison of histopathological features (mean ± SD) between the GP, GP + DBE groups according to the categorization scale given in Table 1

Group	Edema	Vacuolization	Necrosis	Vessels congestion	Inflammation
GP	3.38 ± 0.66	2.95 ± 0.83[1]	1.59 ± 0.75[1]	0.89 ± 0.44	0.21 ± 0.51
GP + DBE	3.29 ± 0.64	2.56 ± 0.97	1.32 ± 0.75	0.93 ± 0.41	0.21 ± 0.56

[1]The existence of significant differences at 95% significance level. Control group is not included, as it had no lesions. Group DBE (GDBE) is also not included because the histological assessment was performed at 7 d after procedure[19]. GP: Group papilla; DBE: Double balloon enteroscopy.

Table 4 Significance levels for the χ^2 test of association between the experimental procedures (GP, GP + DBE) and the frequency of damaged samples

Histopathological parameter	*P* value
Edema	0.73
Vacuolization	0.06[1]
Necrosis	0.09[1]
Vessels congestion	0.80
Inflammation	0.46

[1]The existence of a significantly different frequency of damaged samples in GP and GP + DBE for each histopathological parameter for a 90% confidence ($P < 0.1$).

has been observed in previous studies examining the iatrogenic effects of experimental oral DBE[19]. The differences between groups GP and GP + DBE could be a consequence of the fact that in group GDBE during the oral insertion the balloons were always inflated distally to the duodenal papilla where the pancreatic duct opens into the duodenum, as recommended by some authors[9,22].

The protocol used to monitor post-DBE pancreatitis coincides with the most common protocol in human medicine[2,10,13,22], and was also used in our previous paper[19]. It involves the serological measurement of amylase, lipase and the C-reactive protein (CRP). As a general rule, an increase of amylase and/or lipase higher than twice their basal levels, together with a significant increase of CRP is enough to suspect pancreatitis. There are many potential mechanisms that could hypothetically induce a rise of serum amylase after DBE and some of them have been discussed in previous studies[10,11,13]. For instance, hyperamylasemia has been suggested to occur by the smooth shearing involved while sliding the endoscope and overtube[23] due to its influence in the permeability of the intestine[24]. Thus, in routine DBE in humans amylase levels 24 h after procedure commonly increases up to a bit less than twice baseline levels in a high proportion of patients, 39%[8] 46%[13] and 58%[10]. However, when a relationship between serum amylase and post-endoscopy pancreatitis is suspected, amylase can reach three times the basal levels[25]. Regarding the lipase levels significant increases have also been described in patients at 4 h[9,10,24], 12 h[9] and 24 h[9,10,24] after procedure. It has been suggested that during pancreatic acinar cells injury the sensibility of lipase activity is 82%-100% much better than that amylase activity. Thus, serum lipase activity can increase 2 to 50 times of its upper limit of reference range and remain at such a high level for a longer time than amylase. In previous studies more than 65% of normoamylasemic patients with acute pancreatitis were found to have high lipase activity[26]. Finally, CRP is an indicator sensitive to inflammation but very non-specific regarding the origin of the inflammation. The increased CRP after DBE represents inflammation that could either be mucosal irritation or a clue for suspicion of pancreatitis when combined with hyperamylasemia and hyperlipemia[2,9].

Vacuolization and moderate necrosis in acinar cells was observed in groups GP and GP + DBE, this indicating a relationship between the histological

damage and the degree of manipulation of the duodenal papilla during the procedures. As a general rule, the longer the papilla was in direct contact with the inflated balloons the higher the structural changes found in the pancreas (Tables 3 and 4). From an ethiopatological point of view our histology results may resemble those described in experimental models of pancreatic duct obstruction. These studies showed that pancreatic outflow obstruction alone is sufficient to induce necrotizing pancreatitis[27,28] throughout a sequence of events including acinar cell swelling, vacuole formation, swelling and breakdown of mitochondria, nuclear condensation and rupture of the membranes of organelles[29]. As cell vacuolization may be reversible if the cause is removed this may help to explain the lack of clinical symptoms even in pigs of the GP group[29]. On other hand, in previous experimental studies from our group, where the major and minor duodenal papilla were avoided during DBE oral insertion, no vacuolization was observed in acinar cells. The only injures observed in those animals were related to an ischemic process in the vascular supply to the tail of the pancreas[19].

Regarding the experimental model, it might be argued a probably too long contact time between the overtube's balloon and the papilla in GP and GP + DBE. However, some authors refer to a significant learning curve in acquiring the skills necessary to perform DBE. The results of those authors showed for the first 10 oral-DBE a mean (SD) procedural time of 109 ± 44.6 min[30] or 92.3 ± 38.6 min[31], with a range distance examined between 0 and 665 cm[30] or with a mean speed of less than 1 cm/min for some cases[31]. That indicates that some procedures conducted during the DBE's learning curve have a very low or even null range of explored distance[30,31] without a clear awareness of the position of the inflated overtube's balloon. Other authors also correlate the oral-DBE learning curve with the incidence of hyperamylasemia and pancreatitis[10,23]. In DBE, a gripping force by the overtube balloon is used to support endoscopic insertion, and when an untrained endoscopist applies too much insertion force to the endoscope, the forceful insertion causes slippage of the overtube's balloon and ineffective advancement of the endoscope tip. Finally, in the opinion of several groups DBE should only be performed by an endoscopist with adequate training[15], technical skills and patient volume to maintain their skills[32], a minimum of between 10[31] and 50[33] procedures are required.

In conclusion, during DBE the persistence of the inflated balloon around the area of secretion of the pancreas determines structural damage in the organ and increased levels of biochemical markers (amylase, lipase and CRP). This study in the porcine animal model may help to further understand the potential etiology of post-DBE pancreatitis in humans.

COMMENTS

Background

Disturbance of the pancreatic secretion due to direct trauma to the papilla of Vater is one of the most plausible theories about the etiology of acute pancreatitis after double balloon enteroscopy (DBE).

Research frontiers

In order to avoid potential trauma in the ampullar zone some endoscopists recommend inflating the balloons after passing the ligament of Treitz, although no causal relationship has been found with a lower incidence of hyperamylasemia or reduction in pancreatitis rate.

Innovation and breakthroughs

This is the first study evaluating the manipulation of the duodenal papilla by the inflated balloons during DBE.

Applications

The results of this work support the recommendation of avoiding any contact of the balloons with the duodenal papilla so as to decrease post-DBE pancreatic risk.

Terminology

DBE is a variety of push and pull endoscopy which allows diagnostic and therapeutic actions deep in the small intestine. Although DBE has been considered a reasonably safe technique, articles reviewing complications have been published recently. Among the major complications acute post-procedure pancreatitis is the most severe. Post-DBE pancreatitis is mainly related with the anterograde approach, it may vary from 1%-3% to 12.5%.

Peer-review

Very interesting paper, well designed, dealing with an important clinical problem.

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ORIGINAL ARTICLE

Basic Study

Shear wave elastography results correlate with liver fibrosis histology and liver function reserve

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Author contributions: Qian LX and Feng YH conceived and designed the study; Feng YH, Hu XD, Zhai L and Zu Y developed the methodology; Feng YH, Hu XD, Gui Y and Qian LY acquired the data; Feng YH, Hu XD and Liang S analyzed and interpreted the data; Feng YH, Zhai L, Liu JB and Qian LX wrote, reviewed and/or revised the manuscript; Hu XD, Qian LX and Gui Y provided administrative, technical or material support.

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Abstract

AIM: To evaluate the correlation of shear wave elastography (SWE) results with liver fibrosis histology and quantitative function reserve.

METHODS: Weekly subcutaneous injection of 60% carbon tetrachloride (1.5 mL/kg) was given to 12 canines for 24 wk to induce experimental liver fibrosis, with olive oil given to 2 control canines. At 24 wk, liver condition was evaluated using clinical biochemistry assays, SWE imaging, lidocaine metabolite monoethylglycine-xylidide (MEGX) test, and histologic fibrosis grading. Clinical biochemistry assays were performed at the institutional central laboratory for routine liver function evaluation. Liver stiffness was measured in triplicate from three different intercostal spaces and expressed as mean liver stiffness modulus (LSM). Plasma concentrations of lidocaine and its metabolite MEGX were determined using high-performance liquid chromatography repeated in duplicate. Liver biopsy samples were fixed in 10% formaldehyde, and liver fibrosis was graded using the modified histological activity index Knodell score (F0-F4). Correlations among histologic grading, LSM, and MEGX measures were analyzed with the Pearson linear correlation coefficient.

RESULTS: At 24 wk liver fibrosis histologic grading

was as follows: F0, *n* = 2 (control); F1, *n* = 0; F2, *n* = 3; F3, *n* = 7; and F4, *n* = 2. SWE LSM was positively correlated with histologic grading (*r* = 0.835, *P* < 0.001). Specifically, the F4 group had a significantly higher elastic modulus than the F3, F2, and F0 groups (*P* = 0.002, *P* = 0.003, and *P* = 0.006, respectively), and the F3 group also had a significantly higher modulus than the control F0 group (*P* = 0.039). LSM was negatively associated with plasma MEGX concentrations at 30 min (*r* = -0.642; *P* = 0.013) and 60 min (*r* = -0.651; *P* = 0.012), time to ½ of the maximum concentration (*r* = -0.538; *P* = 0.047), and the area under the curve (*r* = -0.636; *P* = 0.014). Multiple comparisons showed identical differences in these three measures: significantly lower with F4 (*P* = 0.037) and F3 (*P* = 0.032) as compared to F0 and significantly lower with F4 as compared to F2 (*P* = 0.032).

CONCLUSION: SWE LSM shows a good correlation with histologic fibrosis grading and pharmacologic quantitative liver function reserve in experimental severe fibrosis and cirrhosis.

Key words: Liver fibrosis; Histologic grading; Shear wave elastography; Monoethylglycinexylidide test; Experimental study

Core tip: Non-invasive evaluation of liver histology and function reserve is critical for determination of treatment option and prognosis in severe fibrosis and cirrhotic patients. Shear wave elastography (SWE) is a newly emerging elastrographic modality with relatively high resolution and good reproducibility for liver imaging. Lidocaine/monoethylglycinexylidide is also an advanced, laboratory dynamic liver function assay with good diagnostic sensitivity, specificity, and accuracy. This study sheds light on the correlation of SWE imaging results with pharmacologic quantitative liver function for disease severity and function reserve evaluation in patients with severe fibrosis/cirrhosis scheduled for major hepatectomy or liver transplantation.

Feng YH, Hu XD, Zhai L, Liu JB, Qiu LY, Zu Y, Liang S, Gui Y, Qian LX. Shear wave elastography results correlate with liver fibrosis histology and liver function reserve. *World J Gastroenterol* 2016; 22(17): 4338-4344 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i17/4338.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i17.4338

INTRODUCTION

Liver fibrosis is a progressive liver disease characterized by replacement of normal liver parenchymal tissue by fibrotic nonparenchymal tissue with or without concomitant abnormal regenerative nodules[1]. The etiologies of liver fibrosis vary among populations worldwide and mainly include viral hepatitis, especially chronic hepatitis B and C in Eastern and Southeastern Asians, alcoholic liver disease in Westerners, and nonalcoholic fatty liver disease[2]. Aside from portal hypertension, reduced or absent liver function reserve is the major pathophysiologic impairment in severely fibrotic and cirrhotic patients. Diagnosis and severity grading of liver fibrosis, especially the evaluation of liver function reserve, are clinically significant for determining the appropriate treatment modality, such as prioritization of liver transplantation and prediction of prognosis in patients with cirrhosis.

Liver ultrasonography is an non-invasive imaging modality most frequently used for routine liver fibrosis screening but suffers from a low sensitivity and specificity, especially for early liver disease or that complicated by another non-fibrotic disease[3]. A variety of elastrographic techniques, including quasistatic elastrography, transient elastography, acoustic radiation force impulse imaging, shear wave elastography (SWE), and magnetic resonance elastography, have been applied or investigated for quantitative evaluation of liver fibrosis[4]. SWE, also called supersonic shear imaging, is a newly emerging elastrographic modality[5] that has been shown to be clinically beneficial for breast[6], thyroid gland, prostate, musculoskeletal, and liver[7] imaging with relatively high resolution and good reproducibility.

Routine liver function biochemistry assays cannot detect compensated liver insufficiency, and thus, liver-based metabolism tests are normally performed for this purpose. The indocyanine green elimination test is a dynamic, liver metabolism-based function assay mainly used for preoperative bedside evaluation of a patient scheduled for liver resection[8] or transplantation[9]. Lidocaine/monoethylglycinexylidide (MEGX) is also an advanced, laboratory dynamic liver function assay based on the metabolization of lidocaine into MEGX by abundant cytochrome P450 in hepatocytes, with good diagnostic sensitivity, specificity, and accuracy, especially for critically ill patients[10].

The primary objective of this study was to assess the correlation of SWE imaging results and liver histology and MEGX liver function test results in an experimental canine model of liver fibrosis. Specifically, the investigation into the correlation of SWE imaging results with pharmacologic quantitative liver function might aid in the evaluation of the liver function reserve in patients with severe fibrosis/cirrhosis scheduled for major hepatectomy or liver transplantation using a bedside, noninvasive modality rather than an invasive laboratory diagnostic modality.

MATERIALS AND METHODS

Laboratory animals

The study protocol was approved by the Animal Research Committee of Beijing Friendship Hospital, Capital Medical University, in accordance with the

National Institute of Health Guidelines for Laboratory Animal Care and Use. Fourteen healthy laboratory Beagles (Rixin Technology Co., Ltd., Beijing; license No. SCXK[BJ]2011-0007) weighing 6-8 kg, including 6 males and 8 females, were bred at the Center of Laboratory Large Animal in Jilin Sino-Japan Friendship Hospital and housed in individual cages with free access to high-lipid canine feed and tap water containing 1:10 (v/v) ethanol. Under general anesthesia by subcutaneous injection of 3% phenobarbital (1 mg/kg), 12 animals were given a subcutaneous injection of 60% carbon tetrachloride (1.5 mL/kg; Shanghai Chemical Co., Ltd, China) diluted in commercially available olive oil, at a weekly interval for 24 consecutive weeks after the initial injection to induce experimental liver fibrosis[11]. Two animals were given 1.5 mL/kg olive oil alone using the same protocol as controls.

Liver SWE imaging

At 24 wk, the animals were anesthetized and positioned supine with the anterior abdominal wall shaved on the operating table. An Aixplorer color Doppler ultrasound system (SuperSonic Imagine, Aix-en-Provence, France), equipped with a 4-15 MHz probe, was operated by an independent ultrasound technician for SWE imaging. An appropriate right-side intercostal space was located for identifying the optimal liver parenchymal window with gray scale ultrasound imaging. The SWE module was subsequently switched on for elastography of the right lobe parenchyma approximately 1 cm below the liver capsule. Liver stiffness was measured in triplicate from three different intercostal spaces and expressed as the mean elastic modulus (kPa)[12].

Clinical biochemistry assays and MEGX liver function tests

Clinical biochemistry assays were performed at the institutional central laboratory for routine liver function evaluation, including serum total protein, albumin, alanine aminotransferase, asparate aminotransferase, gamma glutamyltransferase, alkaline phosphatase, and bilirubin (total, unconjugated, conjugated). After blood sampling for clinical biochemistry assays, lidocaine hydrochloride (1 mg/kg) was injected through the cephalic vein into the anesthetized animals. The femoral vein was punctured for venous blood sampling (2 mL) at 0, 5, 10, 15, 20, 30, 40, 50, and 60 min after lidocaine injection. Plasma concentrations of lidocaine and its metabolite MEGX were determined using high-performance liquid chromatography repeated in duplicate[13]. A plasma MEGX concentration curve was plotted against time.

Liver biopsy

Percutaneous liver biopsy was performed using an 18-gauge needle by an independent, board-certified interventional ultrasound physician under B-mode ultrasound guidance. Liver biopsy samples were fixed in 10% formaldehyde, and liver fibrosis was graded using the modified histological activity index, *i.e.*, Knodell score (F0-F4), with a higher score indicating more serious liver fibrosis[14].

Statistical analysis

The statistical software package SPSS 11.0 (SPSS Inc., Chicago, IL, United States) was used for statistical analyses. All continuous data are expressed as median ± interquartile range (IQR), and the medians were compared using the Wilcoxon rank-sum test. Multiple comparisons were performed using the Fisher least significance difference test at an adjusted significance level. Correlations among histologic grading, LSM, and MEGX measures were analyzed with the Pearson linear correlation coefficient. A two-sided P value < 0.05 was considered statistically significant.

RESULTS

Correlation of clinical biochemistry with liver histology

All 14 animals survived at 24 wk, and liver fibrosis histologic grading was as follows: F0, n = 2 (control); F1, n = 0; F2, n = 3; F3, n = 7; and F4, n = 2. The liver biochemistry assay results are shown in Table 1. All biochemical measures remained similar among the four histologic groups (P > 0.05); however, the serum albumin level was significantly lower in the F4 group than in the F2 and F0 groups (P = 0.003 and P = 0.021, respectively) but not statistically different among the F3, F2, and F0 groups and between F4 and F3 groups (P > 0.05).

Correlation of SWE with liver histology

Representative SWE images for F0 and F2-F4 are shown in Figure 1A-D. SWE liver stiffness modulus data are shown in Table 2 and exhibited a significant positive correlation with histologic grading (r = 0.835, P < 0.001) (Figure 1E). Specifically, the F4 group had a significantly higher elastic modulus than the F3, F2, and F0 groups (P = 0.002, P = 0.003, and P = 0.006, respectively), and the F3 group also had a significantly higher modulus than the control F0 group (P = 0.039). However, the elastic modulus was similar between the F3 and F2 groups and the F2 and F0 groups (P > 0.05).

Correlation of plasma MEGX pharmacokinetics with liver histology

A plasma MEGX concentration *vs* time plot is shown in Figure 2, and the pharmacokinetics of plasma MEGX are described in Table 3. The four fibrosis grading groups showed no significant differences in all of the pharmacokinetic measures, although a declining trend was observed from F0 to F4, which was statistically significant for plasma MEGX concentrations at 30 min (P = 0.033) and 60 min (P = 0.020) as well as with respect to the area under the curve (P = 0.016).

Table 1 Liver biochemistry assays (median ± IQR) by fibrosis grade (*n* = 14)

Variable	F0 control (*n* = 2)	F2 (*n* = 3)	F3 (*n* = 7)	F4 (*n* = 2)	*P* value
Albumin (g/Dl)	3.9 ± 1.8[1]	3.1 ± 0.5[1]	2.6 ± 0.9[1,2]	1.8 ± 0.5[2]	0.021
Total protein (g/dL)	6.5 ± 1.1	7.0 ± 1.7	6.1 ± 1.2	6.2 ± 1.8	0.639
AST (IU/L)	28 ± 14	21 ± 12	28 ± 15	60 ± 76	0.711
ALT (IU/L)	44 ± 17	43 ± 22	31 ± 28	58 ± 27	0.669
GGT (IU/L)	5.2 ± 2.1	5 ± 1.3	5.2 ± 2.6	6.3 ± 6.2	0.938
ALP (IU/L)	44 ± 25	49 ± 8	61 ± 19	74 ± 31	0.140
TBil (mg/dL)	0.27 ± 0.08	0.24 ± 0.07	0.25 ± 0.11	0.33 ± 0.21	0.865
UBil (mg/dL)	0.13 ± 0.05	0.13 ± 0.06	0.16 ± 0.10	0.17 ± 0.12	0.825
CBil (mg/dL)	0.15 ± 0.03	0.09 ± 0.05	0.10 ± 0.07	0.16 ± 0.09	0.318

[1,2]The two groups with no common letter have a statistically significant difference. ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Asparate aminotransferase; CBil: Conjugated bilirubin; GGT: Gamma-glutamyl transpeptadase; IQR: Interquartile range; TBil: Total bilirubin; UBil: Unconjugated bilirubin.

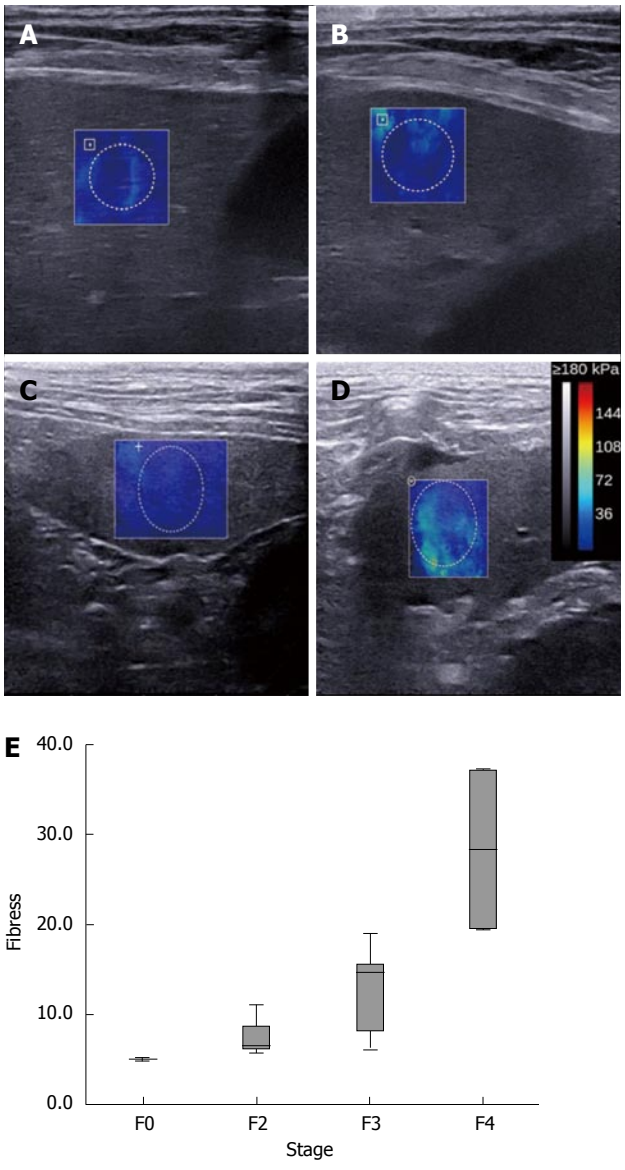

Figure 1 Correlation of shear wave elastography with fibrosis grading. A-D: Representative shear wave elastography images for F0, F2, F3, and F4, respectively; E: Box plot of the elastic modulus (kPa) expressed as median ± IQR against fibrosis grading. IQR: Interquartile range.

Table 2 Shear wave elastography elastic modulus (kPa, median ± IQR) by fibrosis grade (*n* = 14)

Variable	F0 control (*n* = 2)	F2 (*n* = 3)	F3 (*n* = 7)	F4 (*n* = 2)	*P* value
LSM	5.0 ± 0.4[1]	6.5 ± 5.4[1,2]	14.6 ± 9.4[2,3]	28.3 ± 17.8[4]	0.004

[1,2,3,4]The two groups with no common letter have a statistically significant difference. IQR: Interquartile range; LSM: Liver stiffness modulus.

Multiple comparisons showed identical differences in these three measures: significantly lower with F4 (*P* = 0.037) and F3 (*P* = 0.032) as compared to F0 and significantly lower with F4 as compared to F2 (*P* = 0.032), but similar between F3 and F2 and between F2 and F0 (both *P* values > 0.05). LSM was negatively associated with plasma MEGX concentrations at 30 min (*r* = -0.642, *P* = 0.013) and 60 min (*r* = -0.651, *P* = 0.012), time to ½ of the maximum concentration (*r* = -0.538, *P* = 0.047), and the area under the curve (*r* = -0.636, *P* = 0.014).

DISCUSSION

Liver biopsy, usually through the percutaneous approach, is the gold standard diagnostic modality for liver fibrosis/cirrhosis, but this procedure cannot be used and repeated as routine in general clinical practice due to low procedure-associated morbidities, such as bleeding, perforation, and infection[15]. Moreover, histologic grading of fibrosis does not necessarily correlate well with the underlying liver function reserve, but instead may over- or underestimate the disease severity due to the use of a limited liver tissue sample. As an alternative non-invasive diagnostic modality to liver biopsy, liver elastography is an advanced ultrasound or magnetic resonance imaging technique that quantifies liver stiffness by measuring liver tissue distortion (shear wave) and wave transition velocity upon mechanical vibration[16]. Among the elastographic techniques currently available, the

Table 3 Pharmacokinetics (median ± IQR) of plasma monoethylglycinexylidide (n = 14)

Variable	F0 control (n = 2)	F2 (n = 3)	F3 (n = 7)	F4 (n = 2)	P value
$C_{10\ min}$ (ng/mL)	252 ± 31	197 ± 381	99 ± 105	72 ± 38	0.280
$C_{30\ min}$ (ng/mL)	327 ± 56[1]	268 ± 375[1,2]	120 ± 31[2,3]	73 ± 20[3,4]	0.033
$C_{60\ min}$ (ng/mL)	274 ± 71[1]	258 ± 336[1,2]	93 ± 26[2,3]	51 ± 37[3,4]	0.020
C_{max} (ng/mL)	327 ± 56	282 ± 332	143 ± 106	82 ± 22	0.069
t_{max} (min)	30.0 ± 0.0	40.0 ± 20.0	30.0 ± 20.0	17.5 ± 5.0	0.120
$t_{½max}$ (min)	141.3 ± 110.1	151.1 ± 188.3	46.9 ± 50.2	74.9 ± 75.9	0.545
AUC (ng/mL·min)	16644 ± 3139[1]	14421 ± 2515[1,2]	6100 ± 2286[2,3]	3970 ± 1670[3,4]	0.016
K_{max} (ng/mL/min)	43.7 ± 7.2	38.1 ± 83.8	17.7 ± 22.8	6.6 ± 1.5	0.358

[1,2,3,4]The two groups with no common letter have a statistically significant difference. $C_{10\ min}$, $C_{30\ min}$ and $C_{60\ min}$: Concentrations at 10, 30, and 60 min, respectively; C_{max}: The maximum concentration; IQR: Interquartile range; K_{max}: The maximum concentration increase per unit time; t_{max} and $t_{½max}$: Times to the maximum and ½ of the maximum concentration; AUC: Area under the curve.

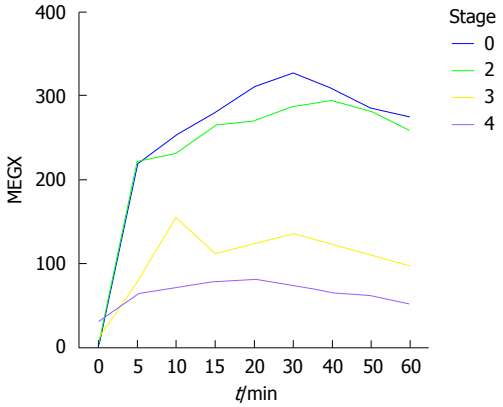

Figure 2 Plasma monoethylglycinexylidide concentration *vs* time plots by fibrosis grade. MEGX: Monoethylglycinexylidide.

FibroScan using transient elastography has been well validated for fibrosis grading in liver fibrosis patients and the results show a good histologic correlation with liver biopsy findings; however, FibroScan measures liver stiffness on a one-dimensional ultrasonograph and also requires an additional designated probe for patients with a narrow rib cage or with complicating fatty liver disease[17]. In contrast, SWE offers a quantitative, real-time, two-dimensional elastography by incorporating an advanced ultrafast imaging technique[5] with a high correlation of histologic fibrosis staging comparable to transient elastography[18]. Our study results add new knowledge to current literature showing that the liver SWE elastic modulus also was well correlated with liver-based drug metabolism in addition to histologic grading. To the best of our knowledge, the present work is the first report regarding correlation of SWE results with fibrosis grading and cytochrome P450-based liver function reserve in experimental liver fibrosis.

Quantitative evaluation of liver function reserve can improve the predictive accuracy for chronic liver disease progression, including chronic hepatitis to liver fibrosis/cirrhosis[19]. The Child-Pugh score[20] and Model for End-Stage Liver Disease score[21] are most often used and based on clinical manifestations and laboratory biochemistry assays; however, these two scales have a variety of confounding factors and only represent a patient's pre-existing long-term liver function reserve rather than an acute change in liver function. The indocyanine green elimination test is the liver function reserve assay most frequently used in general clinical practice; however, its results may be affected by liver perfusion impairment, biliary obstruction, and complicating hypoalbuminemia[22]. MEGX test is a dynamic, quantitative liver function test that measures cytochrome P450 metabolism of lidocaine in metabolically active hepatocytes and is superior to the indocyanine green elimination test with respect to sensitivity, specificity, accuracy, and reproducibility. The MEGX level has been used for preoperative planning of liver resection[23], prediction of post-hepectomy acute liver failure[24], and survival of decompensated cirrhosis patients on the waiting list for liver transplantation[25]. Moreover, quantitative liver function tests including the MEGX test were reported as independent risk factors for improving the predictability of virologic response and disease progression of chronic hepatitis C virus with antiviral treatment[26]. Our results identified three potential measures, especially the area under the curve of the concentration *vs* time plot a sensitive and specific indicator of cytochrome P450 metabolic functionality, which could differentiate severe fibrosis or cirrhosis from mild disease.

SWE has been widely used for the evaluation of liver fibrosis/cirrhosis of multiple etiologies or with complicating comorbidities, including chronic hepatitis[27], liver cancer[28], steatohepatitis[29], and biliary atresia[30]. This two-dimensional elastographic technique offers better performance for assessing liver fibrosis as compared to conventional transient elastography, especially regarding the correlation of the LSM with histologic grading, with cutoff values of 8.0 kPa and 13.1 kPa for F2 and F4, respectively[31]. Our preliminary results demonstrated that severe fibrosis and especially cirrhosis had a higher LSM than moderate liver disease although no statistically significant difference was observed between F2 and F0

or between F3 and F2. Moreover, our results showed that the LSM on SWE was negatively correlated with MEGX test measures, including plasma concentrations at 30 min and 60 min, the time to ½ of the maximum concentration, and with respect to the area under the curve. Quantification of liver stiffness in patients with severe fibrosis or cirrhosis who are scheduled for major hepatectomy or liver transplantation may help estimate the risk of postoperative liver insufficiency with an advantage over pharmacologic liver function assays due to its non-invasiveness and technical reproducibility[32].

In conclusion, our results demonstrated that SWE results are well correlated with the histologic grading of experimental fibrosis. Moreover, SWE results also correlated well with quantitative liver function reserve measurements obtained by the lidocaine metabolite MEGX test. Therefore, it is beneficial to employ SWE for assessment of liver disease severity and function reserve in patients with severe fibrosis or cirrhosis as this modality is non-invasive and reproducible in the clinical setting.

COMMENTS

Background

Liver ultrasonography is a non-invasive imaging modality most frequently used for routine liver fibrosis screening but suffers from a low sensitivity and specificity, especially for early liver disease or that complicated by another non-fibrotic disease. Routine liver function biochemistry assays cannot detect compensated liver insufficiency, and thus, liver-based metabolism tests are normally performed for this purpose.

Research frontiers

Shear wave elastography (SWE) is a newly emerging elastrographic modality that has been shown to be clinically beneficial for liver imaging with relatively high resolution and good reproducibility. Monoethylglycinexylidide (MEGX) is also an advanced, laboratory dynamic liver function assay based on the metabolization of lidocaine into MEGX by abundant cytochrome P450 in hepatocytes, with good diagnostic sensitivity, specificity, and accuracy, especially for critically ill patients.

Innovations and breakthroughs

SWE results are well correlated with the histologic grading of experimental fibrosis and also with quantitative liver function reserve measurements obtained by the lidocaine metabolite MEGX test.

Applications

SWE is beneficial for assessment of liver disease severity and function reserve in patients with severe fibrosis or cirrhosis as this modality is non-invasive and reproducible in the clinical setting.

Peer-review

Interesting paper and topic. In the present study, the authors evaluated the correlation of shear wave elastrography results with liver fibrosis histology and liver function reserve. In general, the manuscript is well-written and the methodology is acceptable. Although the correlation of SWE in patients with various liver conditions have been extensively handled in the literature, this study throws light for the first time on its correlation with quantitative liver function reserve measurements obtained by the lidocaine metabolite MEGX test.

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ORIGINAL ARTICLE

Basic Study

Activation of AMPK/MnSOD signaling mediates anti-apoptotic effect of hepatitis B virus in hepatoma cells

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Abstract

AIM: To investigate the anti-apoptotic capability of the hepatitis B virus (HBV) in the HepG2 hepatoma cell line and the underlying mechanisms.

METHODS: Cell viability and apoptosis were measured by MTT assay and flow cytometry, respectively. Targeted knockdown of manganese superoxide dismutase (MnSOD), AMP-activated protein kinase (AMPK) and hepatitis B virus X protein (HBx) genes as well as AMPK agonist AICAR and antagonist compound C were employed to determine the correlations of expression of these genes.

RESULTS: HBV markedly protected the hepatoma cells from growth suppression and cell death in the condition of serum deprivation. A decrease of superoxide anion production accompanied with an increase of MnSOD expression and activity was found in HepG2.215 cells. Moreover, AMPK activation

contributed to the up-regulation of MnSOD. HBx protein was identified to induce the expression of AMPK and MnSOD.

CONCLUSION: Our results suggest that HBV suppresses mitochondrial superoxide level and exerts an anti-apoptotic effect by activating AMPK/MnSOD signaling pathway, which may provide a novel pharmacological strategy to prevent HCC.

Key words: Hepatitis B virus; Reactive oxygen species; Apoptosis; Manganese superoxide dismutase; AMP-activated protein kinase

Core tip: Hepatitis B virus markedly protected the cells from growth suppression and cell death in the condition of serum deprivation. A decrease of superoxide anion production accompanied with an increase of manganese superoxide dismutase (MnSOD) expression and activity was found in HepG2.215 cells. Moreover, AMP-activated protein kinase activation contributed to the up-regulation of MnSOD. Hepatitis B virus X protein was identified to promote the expression of AMPK and MnSOD.

Li L, Hong HH, Chen SP, Ma CQ, Liu HY, Yao YC. Activation of AMPK/MnSOD signaling mediates anti-apoptotic effect of hepatitis B virus in hepatoma cells. *World J Gastroenterol* 2016; 22(17): 4345-4353 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i17/4345.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i17.4345

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most frequently diagnosed malignant cancers worldwide, while 50% of cases and deaths occurred in China[1]. Chronic hepatitis B virus (HBV) infection has been internationally recognized as one of the major risk factors for the development of HCC[2]. An estimated 350 million people were chronically infected and 600000 hepatitis B-related deaths occurred every year all over the world[3]. Accumulated evidence has shown that HBV proteins, particularly hepatitis B virus X protein (HBx) and surface protein (HBs), are implicated in hepatocyte carcinogenesis[4]. However, the mechanisms underlying HBV-induced malignant transformation remain ambiguous.

Apoptosis, also named programmed cell death, plays a crucial role in the development and homeostasis in normal tissue[5]. Recently, studies have indicated that defect or insufficient apoptosis may contribute to carcinogenesis, tumor progression and resistance of tumor cells to chemo-radiotherapy[6-8]. For that reason, escape of apoptosis has been identified as one of prominent hallmarks of cancer[9]. Reactive oxygen species (ROS), as toxic products of cell metabolism, can cause cell apoptosis by leading to cellular DNA damage and subsequently activating apoptotic signaling pathways[10]. In cancer, tumor niches characterized with poor nutrient and oxygen usually possess oxidative stress with excessive ROS formation[11,12]. Mitochondrial ROS (mtROS) especially superoxide anion, a natural by-product of electron transport chain activity, is the main source of cellular ROS[13]. Thus, decreasing mtROS production to relieve oxidative stress is very important for tumor survival and progression.

Manganese superoxide dismutase (MnSOD), a key antioxidant enzyme, is responsible for scavenging superoxide anion. Liver malignant tumors have been shown to express higher protein level and activity of MnSOD than their benign counterparts[14]. Aggressive tumors possessing invasive phenotype also have a high level of MnSOD, which can facilitate them to reach distant organs[15]. Therefore, increased MnSOD expression and activity may protect cells against apoptosis and offer a growth advantage, thereby acquiring a more aggressive phenotype.

The expression of MnSOD can be modulated by many molecular factors at transcription, translation and posttranslational modifications levels, for example, p53, Sp1, and NF-κB[16-18]. AMP-activated protein kinase (AMPK) is also reported to act as a new regulator of MnSOD expression in endothelial cells[19]. Moreover, AMPK activation is associated with protection of hepatocytes against oxidative stress[20].

Based on the aforementioned studies, we investigated the effect of HBV on the growth and survival of HepG2 cells, and explored the underlying molecular mechanisms. Herein, we demonstrated that HBV protected HepG2 cells from growth suppression and apoptosis in the condition of serum deprivation. Furthermore, AMPK activation-induced up-regulation of MnSOD contributed to the resistance of HBV-integrated HepG2 cells to apoptosis caused by superoxide, which could explain in part HBV-induced hepatocellular cell malignant transformation in the context of growth factor withdrawal.

MATERIALS AND METHODS

Cell culture

The human hepatoma cell line HepG2 was obtained from Cell Bank of Chinese Academy of Sciences where it was authenticated. HepG2.215 cell line, which was derived from HepG2 cells by integrating HBV genome and persistently produced HBV, was kindly provided by Prof. Erwei Song (Sun Yat-sen Memorial Hospital of Sun Yat-sen University, China). All of the cell lines were maintained in DMEM (Gibco, Gaithersburg, MD, United States) supplied with 10% fetal bovine serum and 1% penicillin/streptomycin, and incubated at 37 ℃ in a humidified incubator with 5% CO_2.

Regents

The HiPerFect transfection reagent was obtained from QIAGEN (QIAGEN, Carson City, CA). Antibodies of AMPKα and phospho-AMPKα (Thr^{172}) were purchased from Cell Signaling (Cell Signaling Technology, MA). Anti-MnSOD antibody was from BD (BD Pharmingen, San Diego, CA, United States). Antibody of HBx (anti-HBx) was obtained from Abcam (Abcam, Cambridge, UK). AICAR, Compound C and anti-β-actin were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, United States).

Cell viability assay

Cells were seeded in 24-well plates in quadruplicate. After indicated treatments, cells viability was determined with 3-[4,5-dimethylthiazol-2-yl]-2,5-dephenyl tetrazolium bromide (MTT) (Sigma, MO) following the manufacture's protocol. Absorbance was measured at a wavelength of 570 nm.

Cell apoptosis assay

Cells were prepared as described elsewhere[21]. AnnexinV and propidium iodide (KeyGEN BioTECH, Nanjing, China) were added for incubation in the dark for 15 min at 4 ℃, and then cells were analyzed with a flow cytometer (Gallios, Beckman).

Mitochondrial superoxide anion detection

Measurements of mitochondrial superoxide anion formation in cells were performed as previously described[22]. In brief, HepG2 and HepG2.215 cells were incubated with 5 μmol/L MitoSOX (Invitrogen, Carlsbad, CA) for 20 min at 37 ℃. Cells were digested using EDTA (Invitrogen), and then washed three times using HBSS with Ca/Mg (Invitrogen). Mean fluorescent intensity was measured by flow cytometry (Gallios, Beckman).

MnSOD activity measurement

MnSOD activity was measured with a commercial SOD kit (Cayman Chemical) according to the manufacturer's protocol. Briefly, 1 mmol/L potassium cyanide was added in order to inhibit Cu/Zn-SOD and extracellular SOD, thus only MnSOD activity was detected. $O2^-$ was generated by adding hypoxanthine/xanthine oxidase and detected with tetrazolium salt through reading the absorbance at 450 nm.

RNA interference

The siRNAs for silencing AMPK, MnSOD and HBx genes as well as scrambled siRNA were purchased from Ribobio (Guangzhou, China). Transfection with synthetic siRNAs was performed with HiPerFect (QIAGEN, Carson City, CA) according to the manufacturer's instructions. The sense sequences of double-strand siRNA were as follows: siAMPK, 5'-UGCCUACCAUCUCAUAAUATT-3'; siMnSOD, 5'-GGAGAAUGUAACUGAAAGATT-3'; siHBx-1, 5'-CCGACCUUGAGGCAUACUUdTdT-3'; siHBx-2, 5'-UGUGCACUUCGCUUCACCUTT-3'.

Western blot analysis

Western blot analysis was performed as described previously[23]. Antibodies for MnSOD, AMPK, Phospho-AMPK and HBx were used at 1:1000 dilution. Antibody for β-actin was used at 1:10000 dilution. Bound antibody was visualized using HRP-conjugated secondary antibodies.

Statistical analysis

All data are expressed as mean ± standard deviation (SD). SPSS 13.0 software was used for one-way analysis of variance (ANOVA) and *t*-test in all statistical analyses (SPSS, Chicago, IL, United States). A *P* value less than 0.05 was considered statistically significant.

RESULTS

HepG2.215 is more tolerant to serum-deprivation environment

In order to assess the effect of HBV on the proliferation of HepG2 cells, we employed the HepG2.215 cell line which was derived from the HepG2 cell line and persistently produced HBV. We found that the HepG2.215 cell line showed faster growth kinetics compared with the HepG2 cell line on days 4 and 6 after serum depletion (Figure 1A). Moreover, the number of apoptotic HepG2 cells was significantly increased on days 4 and 6 compared with that of HepG2.215 cells. In contrast, the number of apoptotic HepG2.215 cells stayed at a much lower level at all testing time points (Figure 1B and C). These data suggest that HBV proteins may protect HepG2.215 cells against apoptosis induced by serum depletion.

Decreased mitochondrial superoxide level may be due to increased MnSOD expression and activity

To explain the different anti-apoptotic ability of the two cell lines, we investigated the production of mitochondrial superoxide which is a well-known killer of cells[10]. Decreased mitochondrial superoxide level was found in the HepG2.215 cell line (Figure 2A). Since MnSOD is the regulator of mitochondrial superoxide, we therefore detected the expression and activity of MnSOD in the two cell lines. As shown in Figure 2B and C, both the expression and activity of MnSOD in HepG2.215 cells were higher than those of HepG2 cells.

MnSOD mediates the apoptotic resistance of HepG2.215 cells

To further verify the role of MnSOD in the apoptotic resistance of HepG2.215 cells, the MnSOD siRNA was synthesized. Western blot analysis revealed that MnSOD siRNA specifically knocked down MnSOD in HepG2.215 cells (Figure 3A). Knockdown of MnSOD decreased cell viability and increased mitochondrial

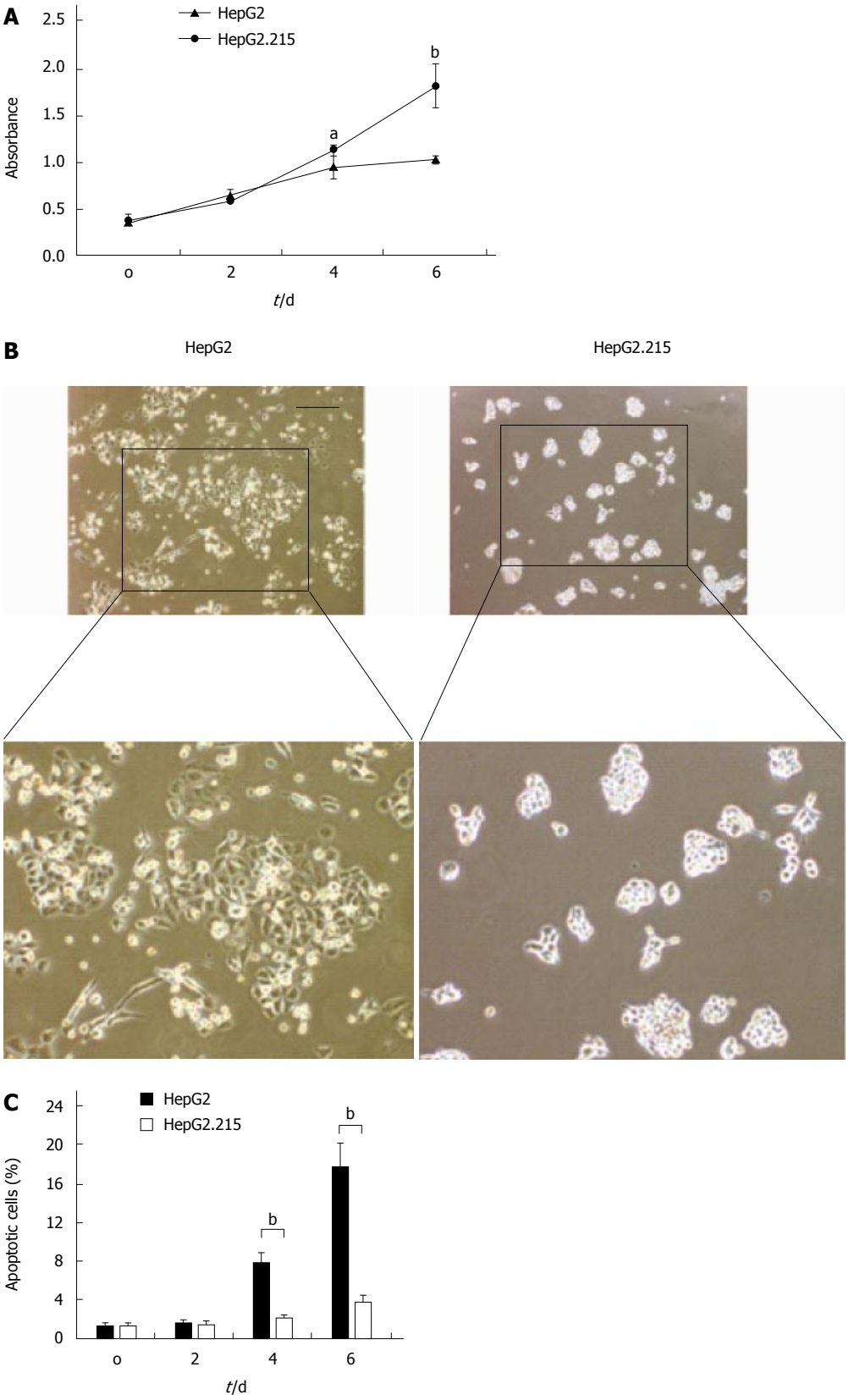

Figure 1 Resistance of HepG2.215 cells to apoptosis. A: Typical photographs of HepG2 and HepG2.215 cells cultured for 6 d after serum depletion. The data are representative of three independent experiments; B: MTT assay for the viability of cells cultured for 2, 4 and 6 d after serum depletion. Data represent absorbance at 570 nm and are shown as mean ± SD of quadruplicates. $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* HepG2 cells group. The data are representative of three independent experiments; C: In parallel experiments, samples were subjected to FITC-Annexin V/propidium iodide staining and the quantitative analysis of apoptotic cells was performed using flow cytometry. Quantification of apoptotic cells is shown as mean ± SD of triplicates.

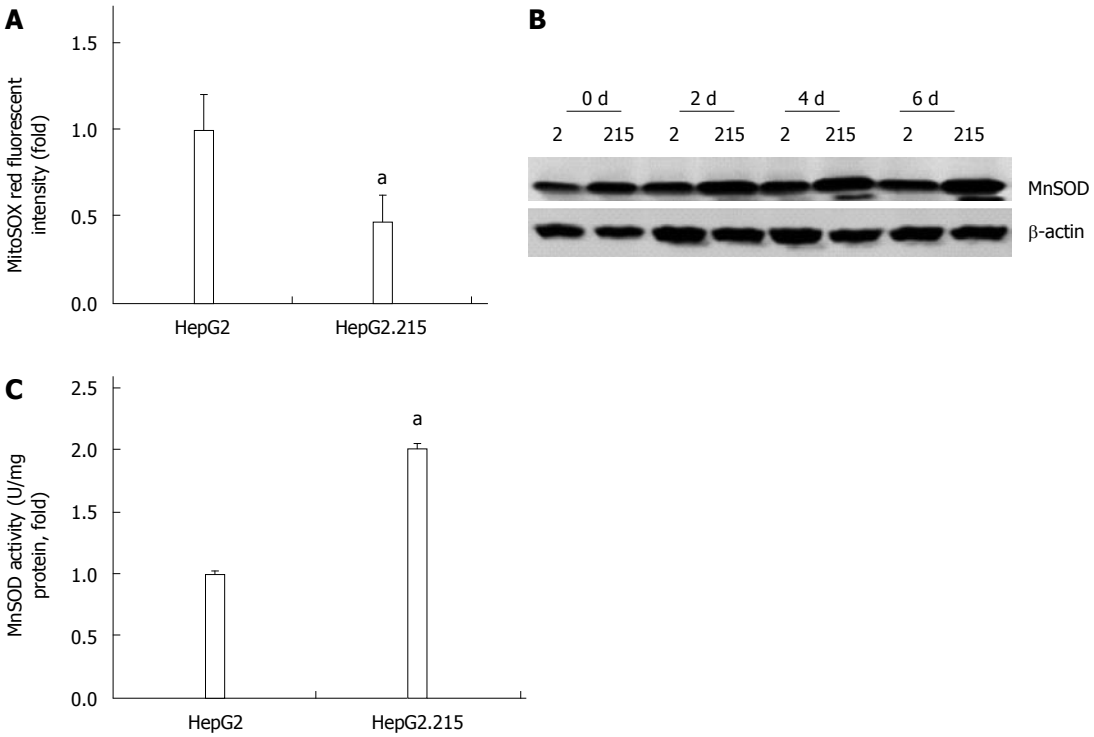

Figure 2 Decreased mitochondrial superoxide production is accompanied by increased MnSOD expression and activity in HepG2.215 cells. A: Mitochondrial superoxide level was decreased in HepG2.215 cells. Cells were cultured in serum-free medium for 6 d. Mitochondrial superoxide anion formation was measured by flow cytometry and MitoSOX. Quantification of mitochondrial superoxide anion is shown as mean ± SD of triplicates. $^{a}P < 0.05$ *vs* HepG2 cell group; B: Cell lysates were subjected to Western blot analysis. The protein level of MnSOD was detected; C: MnSOD activity was measured with a commercial SOD Assay Kit. Quantification of MnSOD activity is shown as mean ± SD of triplicates. $^{a}P < 0.05$ *vs* HepG2 cells group.

superoxide formation and the number of apoptotic HepG2.215 cells (Figure 3B-D), which suggests that MnSOD plays a critical role in apoptotic resistance of HepG2.215 cells.

AMPK activation contributes to up-regulation of MnSOD in HepG2.215 cells

To figure out the upstream factor involving the modulation of MnSOD, AMPK was investigated. We showed the protein levels of p-AMPK and AMPK were increased in HepG2.215 cells (Figure 4A). Both knockdown of AMPK and treatment with AMPK inhibitor Compound C reduced the expression of MnSOD (Figure 4B and C). Conversely, AMPK activator AICAR increased the expression of MnSOD (Figure 4C). Furthermore, the expression of p-AMPK, AMPK and MnSOD was inhibited by HBx knockdown (Figure 4D). These results suggest that HBV up-regulates MnSOD *via* AMPK.

DISCUSSION

As a major cause for HCC development, HBV can promote HCC in many ways, including enhancing host chromosomal stability, inducing inflammation-mediated immune escape, regulating epigenetic modification or altering the expression of oncogenes and tumor-suppressor genes[24]. Due to these internal changes, hepatoma cells acquire the capacity of fast growth, anti-apoptosis and metastasis[25,26]. In this study, we confirmed that HBV-integrated HepG2 cells exerted survival benefit compared with its parent cell line HepG2 in the serum-deprivation condition which can to some extent mimic the adapation of tumor cells to adverse growth conditions. In line with previous studies, we also found that HBV conferred HepG2 cells resistance to apoptosis[26,27]. Our data suggest that HBV apparently acts to promote the growth and viability of hepatoma cells in growth factor-restricted conditions.

An increased level of ROS by creating a potentially toxic environment to the cells represents a critical mechanism underlying cell death[28]. Superoxide anion is the precursor of other ROS such as H_2O_2 and peroxynitrite, and because of that the organelles most vulnerable to oxidative stress are the mitochondria[29]. MnSOD is an essential antioxidant enzyme in the mitochondrion that acts on superoxide anion[30]. Here, we showed that HBV reduced the level of superoxide anion. Consistently, the expression and activity of MnSOD were up-regulated in HBV-integrated HepG2 cells. This result was supported by the finding of a previous study that in patients with HBV infection, there was an average 5-fold rise of serum MnSOD[31].

The expression and activity of MnSOD are not static in different tumorigenesis stages. For transformed phenotype, MnSOD levels were maintained at a low level and could directly potentiate mitochondrial

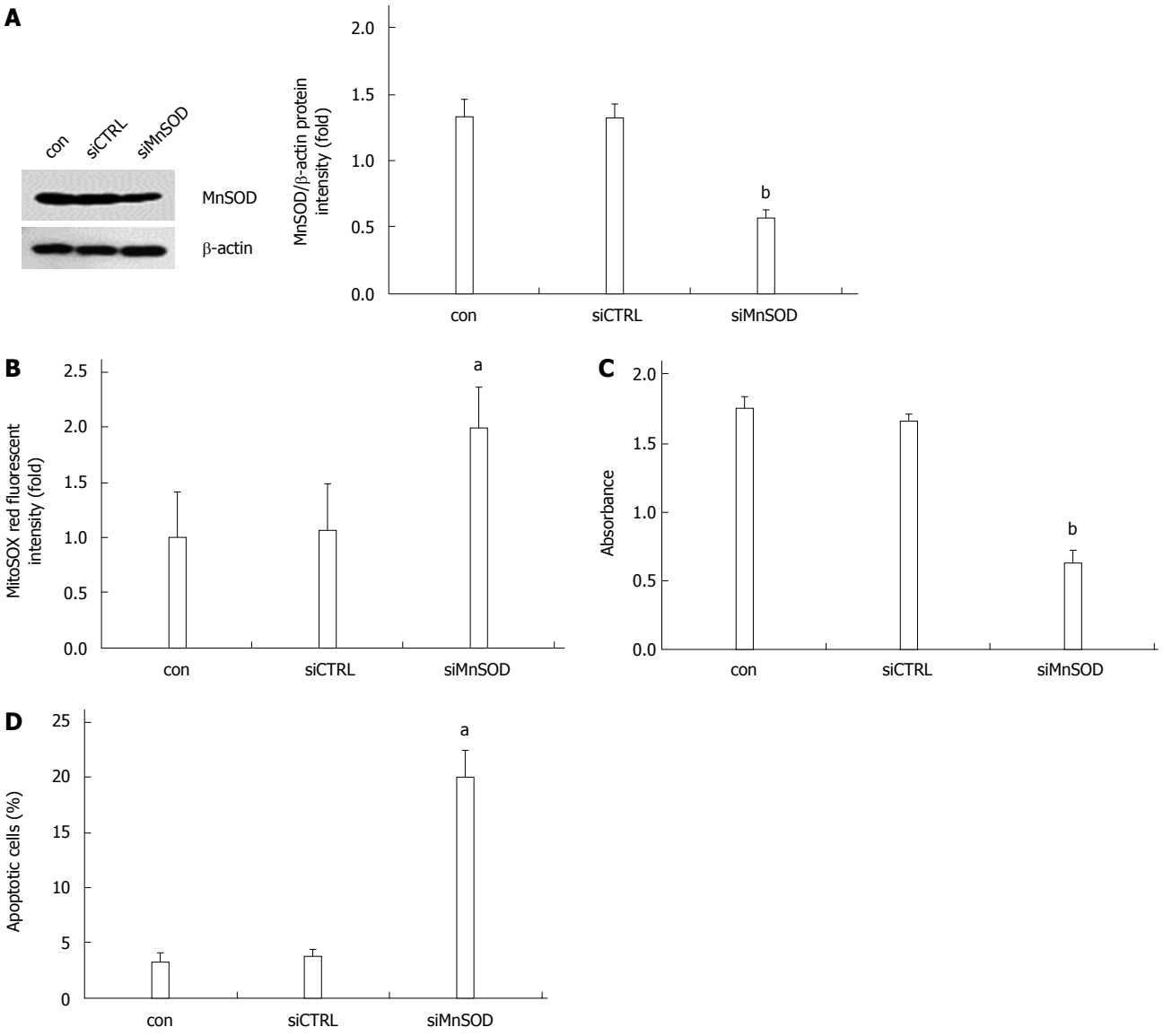

Figure 3 MnSOD contributes to decreased mitochondrial superoxide and apoptotic cells in HepG2.215 cells. A: After 6 d, the interference effect of MnSOD siRNA (siMnSOD) was analyzed by Western blot analysis. MnSOD siRNA (siMnSOD) or non-specific siRNA (siCTRL) was transfected into HepG2.215 cells for 12 h before serum depletion; B: After 6 d, cells were harvested for quantification of mitochondrial superoxide anion formation by flow cytometry. In parallel, (C) cell viability and (D) apoptotic cells were separately determined by MTT assay and flow cytometry. $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* siCTRL group.

defects, leading to gene mutations. For acquiring a more aggressive phenotype, enhanced MnSOD activity may protect cells against mitochondrial injury, thereby conferring a growth advantage to the cancer cells[16]. The present study demonstrated that knockdown of MnSOD increased the production of superoxide anion and the apoptosis of HepG2.215 cells, which indicated that MnSOD protected hepatoma cells against apoptosis by detoxing superoxide anion, and conferred a growth advantage to those cells. However, since the function of MnSOD is to convert diffusion-restricted and mild-toxicant superoxide anion to freely diffuse and strong-toxicant H_2O_2, which means that increased MnSOD may enhance the production of more toxicant H_2O_2, the mechanism of modulation of tumor cell survival by MnSOD seems confusing. It has been reported that HBx expressing cell line showed significantly reduced sensitivity to H_2O_2-induced cell death, and the level of intracellular ROS did not elevate in HBx expressing cell line after exposure to H_2O_2 in the medium[32]. Based on these findings, we speculate that HBV-infected cells may express relatively high amounts of catalase, they would be able to counteract the cytotoxic effects of peroxide, and thus the outcome of increased MnSOD activity would more likely reflect the capacity of MnSOD to reduce levels of oxygen radicals. Unexpectedly, the level of catalase in HBV-related hepatocellular carcinoma specimens was lower than that of surrounding non-tumor tissues[33]. Thus, further investigation is required to explain the tolerance of HBV-infected cells to H_2O_2-induced cell apoptosis, which will be helpful for understanding the mechanism of MnSOD-modulated tumor cell survival.

AMPK, a serine/threonine protein kinase, is well

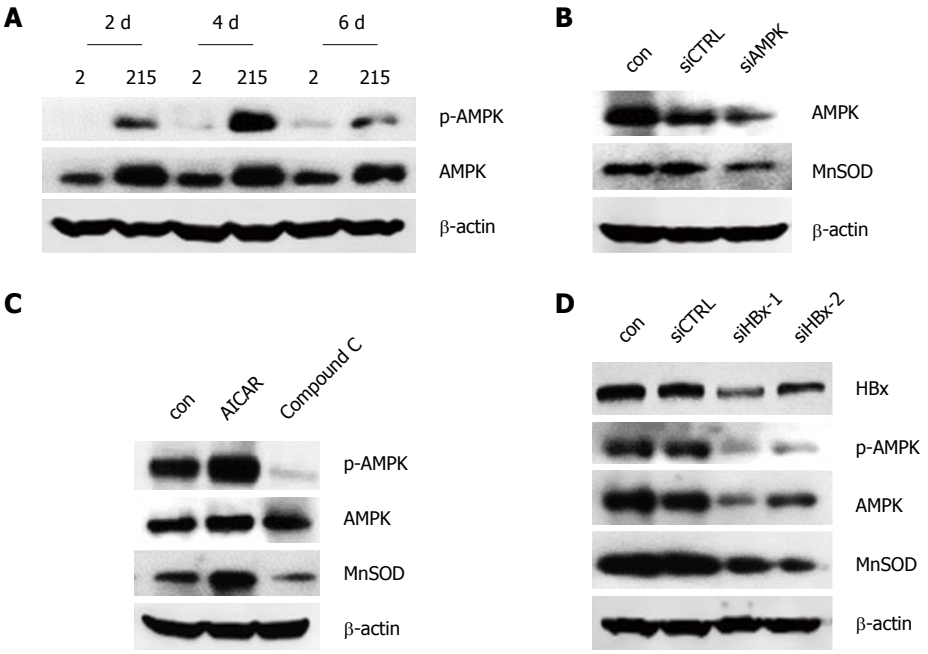

Figure 4 AMPK activation is required for MnSOD expression in HepG2.215 cells. A: Cell lysates were subjected to Western blot analysis. The protein levels of AMPK and p-AMPK were detected; B: Cells were transfected with AMPK siRNAs (siAMPK) or a nonspecific control siRNA (siCTRL) for 6 d. The protein levels of AMPK and MnSOD were detected by Western blot analysis; C: Cells were subjected to 1 mM AMPK activator AICAR or inhibitor compound C for 6 d. The protein levels of AMPK, p-AMPK and MnSOD were detected by Western blot analysis; D: Cells were transfected with HBx siRNAs (siHBx) or a nonspecific control siRNA (siCTRL) for 6 d. The protein levels of AMPK and MnSOD were detected by Western blot analysis.

known for its role in controlling energy metabolism. Recently, it comes into focus because of its potential roles in regulating other signaling pathways, such as regulating oxidative stress[34]. Studies have reported that activation of AMPK by AICAR, or overexpression of constitutively activated AMPK suppressed O_2^- production in human neutrophils or HUVECs[35,36]. A similar observation was also found in HepG2 cells, which showed that AA+ iron-induced reactive oxygen species generation was inhibited by isorhamnetin through AMPK activation[20]. These studies indicate that AMPK appears to be the key factor for cellular function protection in the presence of oxidative stress. Emerging evidence suggests that AMPK inhibits oxidant production by decreasing the expression of NADPH oxidases or increasing the expression of UCP-2 as well as MnSOD[19,35,36]. In the present study, HBV-integrated HepG2 cells displayed elevated AMPK protein level, which remains consistent with the expression of MnSOD. By utilizing a specific siRNA, or a selective agonist (AICAR) and antagonist (compound C) of AMPK, we observed that knockdown of AMPK and compound C resulted in the reduction of MnSOD protein level. Moreover, activation of AMPK by AICAR up-regulated the expression of MnSOD. Taken together, these findings demonstrate that AMPK is responsible for the up-regulation of MnSOD expression in HBV-integrated HepG2 cells.

Additionally, numerous studies have shown that HBx protein serves as a transactivator in the pathogenesis of HCC through regulating cell transformation, apoptosis and cellular immune system[37-39]. In our study, HBx was identified as the active ingredient of HBV proteins to promote the expression of AMPK and MnSOD. This is consistent with previous investigation reported by Severi *et al*[32] that HBx expressing cell line is more resistant to ROS-induced cell apoptosis than HBsAg expressing cell line. These data suggest that HBx may alleviate oxidative stress by up-regulating AMPK/MnSOD axis to maintain "normal" live cancer cell functions.

In summary, our current study demonstrates that HBV suppresses mitochondrial superoxide level and exerts an anti-apoptotic effect by activating AMPK/MnSOD signaling pathway in HBV-infected HepG2 cells. These findings may provide a novel mechanism involved in HBV-triggered carcinogenesis, and therefore might be useful in the design of new pharmacological approaches to prevent HCC.

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COMMENTS

Background

Chronic hepatitis B virus (HBV) infection is one of the major risk factors for the development of hepatocellular carcinoma (HCC). However, the mechanisms

underlying HBV-induced HCC remain ambiguous. Recently, accumulated evidence has shown that escape of apoptosis may contribute to carcinogenesis.

Research frontiers

Previous experiments have revealed that liver malignant tumors and patients with HBV-infection express higher protein level of manganese superoxide dismutase (MnSOD) than their counterparts. Here, the authors showed that high expression of MnSOD protected hepatoma cells against apoptosis by detoxing superoxide anion, and conferred a growth advantage to those cells. These results explain how HBV offers a survival benefit to hepatoma cells.

Innovations and breakthroughs

This is the first study to demonstrate that HBV protects hepatoma cells against apoptosis *via* AMPK/MnSOD signaling pathway. HBV markedly protected the cells from growth suppression and cell death in the condition of serum deprivation. A decrease of superoxide anion production accompanied with an increase of MnSOD expression and activity was found in HepG2.215 cells. Moreover, AMPK activation contributed to the up-regulation of MnSOD. HBx protein was identified to promote the expression of AMPK and MnSOD. These results provide further evidence for the role of HBV as a major cause of HCC development *via* an anti-apoptosis mechanism involving activation of AMPK/MnSOD signaling pathway.

Applications

The present results suggest that HBV suppresses mitochondrial superoxide level and exerts an anti-apoptotic effect by activating AMPK/MnSOD signaling pathway, which may be useful in the design of new pharmacological approaches to prevent HCC.

Peer-review

In this study, Li *et al* aimed to investigate the anti-apoptotic capability of the hepatitis B virus in the HepG2 hepatoma cell line by suppressing mitochondrial superoxide levels. Generally, their findings seem to be interesting, anyway it should be validated in different cell lines, such as HepG2.117.

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ORIGINAL ARTICLE

Basic Study

Inhibitory effect of miR-125b on hepatitis C virus core protein-induced TLR2/MyD88 signaling in THP-1 cells

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Author contributions: Peng C and Yang DL designed the research; Lu MJ instructed on the whole study; Peng C, Wang H and Zhang WJ performed the research; Tong QX contributed new reagents or analytic tools; Peng C and Jie SH analyzed the data; Peng C, Lu MJ and Yang DL wrote the paper.

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Conflict-of-interest statement: We declare that we have no financial or personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and company. The authors declare that there is no conflict of interests regarding the publication of this paper.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at dlyang@hust.edu.cn. Participants gave informed consent for data sharing. No additional data are available.

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Abstract

AIM: To investigate the role of miR-125b in regulating monocyte immune responses induced by hepatitis C virus (HCV) core protein.

METHODS: Monocytic THP-1 cells were treated with various concentrations of recombinant HCV core protein, and cytokines and miR-125b expression in these cells were analyzed. The requirement of Toll-like receptor 2 (TLR2) or MyD88 gene for HCV core protein-induced immune responses was determined by the transfection of THP-1 cells with gene knockdown vectors expressing either TLR2 siRNA or MyD88 siRNA. The effect of miR-125b overexpression on TLR2/MyD88 signaling was examined by transfecting THP-1 cells with miR-125b mimic RNA oligos.

RESULTS: In response to HCV core protein stimulation, cytokine production was up-regulated and miR-125b expression was down-regulated in THP-1

cells. The modulatory effect of HCV core protein on cellular events was dose-dependent and required functional TLR2 or MyD88 gene. Forced miR-125b expression abolished the HCV core protein-induced enhancement of tumor necrosis factor-α, interleukin (IL)-6, and IL-10 expression by 66%, 54%, and 66%, respectively ($P < 0.001$), by inhibiting MyD88-mediated signaling, including phosphorylation of NF-κBp65, ERK, and P38.

CONCLUSION: The inverse correlation between miR-125b and cytokine expression after HCV core challenge suggests that miR-125b may negatively regulate HCV-induced immune responses by targeting TLR2/MyD88 signaling in monocytes.

Key words: miR-125b; Hepatitis virus C; Toll like receptor 2; Monocytes; Innate immunity

Core tip: Increasingly many studies have shown that microRNAs are critical regulators of the innate immune response. Many anti-pathogen pathways, including the pattern recognition Toll-like receptor (TLR)-mediated signaling pathway, are known to be regulated by a network of microRNAs. Here we investigated the possible role of miR-125b in regulating the monocyte immune responses induced by HCV core protein through TLR2/MyD88 signaling. Our findings indicated that miR-125b may function as a negative regulator of HCV-induced cellular events, which may provide insight into the role of miR-125b in the innate immune responses of monocytes to HCV.

Peng C, Wang H, Zhang WJ, Jie SH, Tong QX, Lu MJ, Yang DL. Inhibitory effect of miR-125b on hepatitis C virus core protein-induced TLR2/MyD88 signaling in THP-1 cells. *World J Gastroenterol* 2016; 22(17): 4354-4361 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i17/4354.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i17.4354

INTRODUCTION

Hepatitis C virus (HCV), one of the primary causes of chronic hepatitis, end-stage cirrhosis, and hepatocellular carcinoma (HCC), contributes to an estimated 200 million chronic infections worldwide and 3-4 million or more each year[1]. HCV is a small enveloped RNA virus belonging to the Flavivridae family. The HCV genome encodes a polyprotein precursor of approximately 3000 amino acids that is processed by host and viral proteases into at least 10 different proteins. The viral core protein, a major cleaved product of HCV polyprotein by host peptidases, is known for its role in mediating HCV-induced pathogenesis. The RNA-binding HCV core protein is involved not only in the formation of the viral nucleocapsid but is capable of directly interacting with various host cell components, resulting in cellular dysfunction and carcinogenesis[2].

HCV not only infects hepatocytes, but it replicates in other immune cells, including B cells, T cells, NK cells and monocytes/macrophages[3,4]. It has been postulated that HCC is a consequence of prolonged liver injuries induced by chronic and persistent HCV infections. Numerous reports have indicated that defective host innate and adaptive immune systems are the major culprits facilitating the inability of the host to eradicate viruses[5]. While there is clear evidence that the synthesis of anti-viral cytokines, interferon (IFN)-α and IFN-γ were defective in chronic HCV infected patients[6], various others cytokines, such as tumor necrosis factor (TNF)-α, interleukin(IL)-6 and IL-10, were excessively expressed in patients with HCV viremia[7]. These skewed cytokine productions have been suggested to play a key role in liver injury, viral persistence and the progression to chronic HCV infection[8].

Monocytes/macrophages function as both innate immune cells by killing pathogens directly, and producing anti-pathogen cytokines and as adaptive immune cells by presenting antigens to other immune cells to produces antibodies. Although monocytes/macrophages dysfunction has been documented in patients with chronic HCV infection, particularly defective response to TLR ligand stimulation[9], the precise mechanism as how these cells are involved in the pathogenesis remains unclear.

MicroRNAs are highly conserved endogenous non-coding small RNAs that act as translational repressors to regulate a wide range of biological processes[10]. MicroRNAs have also been implicated in the pathogenesis, diagnosis and therapeutic aspects of HCV infections[11]. Recent studies have shown that several microRNAs, including miR-21, miR-146a and miR-155, were involved in regulation of virus-host interactions and may play important roles in the pathogenesis of HCV infection[12-14].

Since miR-125b is highly expressed in peripheral blood mononuclear cells, particularly in monocytes/macrophages[15], and its expression was found to be inversely associated with better treatment outcome of chronic HCV infection[16], we explored the role of miR-125b in modulating HCV-induced events in monocytic THP-1 cells. Here we show that miR-125b expression was negatively correlated with HCV core-induced cytokines expression in a TLR2/MyD88-dependent manner.

MATERIALS AND METHODS

Cell culture and reagents

The THP-1 human monocytic leukemia cell line was obtained from the Institute of Biochemistry and Cell Biology at the Shanghai Institute for Biological Sciences

and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 IU/mL), and streptomycin (100 mg/mL). Bacterial HCV core recombinant protein was obtained from Immune Technology Co., China. Stability-enhanced miR-125b RNA oligonucleotide, miR-125b mimic (miR-125bM), and the control non-targeting RNA oligonucleotide were purchased from GenePharma.

RNA extraction and real-time quantitative reverse transcription-polymerase chain reaction analyses of cellular and miRNA genes

Total RNA (cellular and miRNA) was extracted from 1-3 × 10^6 THP-1 cells using TRIzol reagent (Life Technologies). For cellular gene expression, first strand cDNA and real-time quantitative polymerase chain reaction (RT-qPCR) analyses of TLR2 and MyD88 were performed according to the manufacturer's instructions (Kakara). The following PCR primers were used in this study: TLR2 (F: 5'-TGGCATGTGCTGTGCTCTGTT-3'; R: 5'-AGCTTTCCTGGGCTTCCTTTT-3'); MyD88 (F: 5'-CTCCTCCACATCCTCCCTTC-3'; R: 5'-GCTTGTGT-CTCCAGTTGCC-3'); internal control β-actin (F: 5'-CA-CGATGGAGGGGCCGGACTCATC-3'; R: 5'-TAAAG-ACCTCTATGCCAACACAGT-3'). The reaction conditions for PCR were 95 ℃ for 60 s, 53 ℃ for 60 s, and 72 ℃ for 60 s for 40 cycles, followed by an extension at 72 ℃ for 8 min. The relative gene expression of TLR2 and MyD88 was calculated using 2^{-ddCt}, where dd*Ct* = (*Ct*[gene]-*Ct*[β-actin]) and *Ct* is the crossing threshold value returned by the PCR instrument for each gene amplification.

To measure miRNA expression, total RNA was reversely transcribed at 16 ℃ for 30 min, 42 ℃ for 30 min and 85 ℃ for 5 min using miRNA gene specific primer (has-miR-125-5p and U6 snRNA NR_004394; Applied Biosystems). MicroRNA expression was determined by quantitative PCR using TaqMan Universal PCR System (Life Technologies) in 20 μL reactions, containing 1 μL TaqMan probes. The reaction was performed at 95 ℃ for 10 min, followed by 95 ℃ for 15 s and 60 ℃ at 60 s for 40 cycles. Expression of target genes was calculated as relative to that of the internal U6 snRNA.

Short interfering RNA knockdown vectors and transfections

TLR2-short interfering RNA (TLR2-siRNA) and MyD88-siRNA were constructed as previously described[12,13]. In brief, double-stranded oligonucleotides corresponding to the 164-182 position of TLR2 gene (NM_003264) or the 880-898 position of the MyD88 gene (NM_001172566) sequences were selected according to BLOCK-iT™ RNA Designer (Life Technologies) and cloned into Bsa I/Sac I sites of the pBSilence1.1 plasmid (Sirui Biologicl Co.). The recombinant plasmids were verified by sequencing from both ends. Silencing of TLR2 or MyD88 in THP-1 cells was performed using Lipofectamine 2000 (Life Technologies) and 50 nmol/L siRNA vector DNA according to the manufacturer's protocol. Cells transfected with empty pBSilence1.1 vector was used as a transfection control. Twenty-four hours after transfection, the cells were analyzed for RNA (by RT-qPCR) or protein (by Western blot) expression.

Transfection of miR-125b mimic or control RNA oligos was similarly performed using Lipofectamine 2000. Six hours post transfections, the cells were treated with 5 μg/mL HCV core protein and incubated for an additional 6 h at 37 ℃ prior to further assays.

Enzyme-linked immunosorbent assay

The levels of TNF-α, IL-6 and IL-10 in the supernatants were measured using a human ELISA kit (elabscience) according to the manufacturer's instructions. The detection ranges of TNF-α, IL-6 and IL-10 are > 8 pg/mL, > 4 pg/mL, and 7.813-500 pg/mL, respectively.

Western blot analysis

Cell lysates were prepared in a sodium dodecyl sulfate (SDS) sample buffer [62.5 mmol/L Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mmol/L 1,4-dithiothreitol, and 0.1% bromophenol blue] containing a mixture of protease and phosphatase inhibitors. Lysates (25 μg) were separated on 12% acrylamide gels and transferred to a nitrocellulose membrane in Tris-glycine buffer containing 20% methanol. The membranes were then blocked with 5% milk in 1 × Tris-buffered saline and 0.1% Tween-20 for 1 h at room temperature and probed with various diluted primary monoclonal antibodies overnight at 4 ℃ with constant rocking. After extensive washing, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin G (Boster Biological Tech) at room temperature for 2 h. Protein expression was determined using a chemiluminesence method (Thermo Scientific). Antibodies recognizing TLR2, total NF-κBp65, phosphor-NF-κBp65 (Abcam), MyD88(Bioworld), phospho-ERK (Cell Signaling), and phospho-P38 (Santa Cruz Biotech) were used to probe the membranes. To control protein loading, the blots were stripped and re-probed with an anti-glyceraldehyde 3-phosphate dehydrogenase antibody (Xianzhi Lifescience).

Statistical analysis

Data are shown as mean ± SEM. Unpaired two-tailed Student's *t*-test was used to compare two independent groups. Graphpad 5.0 software (San Diego, CA) was used for all statistical analyses, and *P*-values < 0.05 were considered statistically significant.

RESULTS

HCV core protein up-regulates TNF-α, IL-6 and IL-10 expression and down-regulates miR-125b expression in THP-1 cells

We first tested whether treatment with HCV core protein regulates miRNA expression. THP-1 was

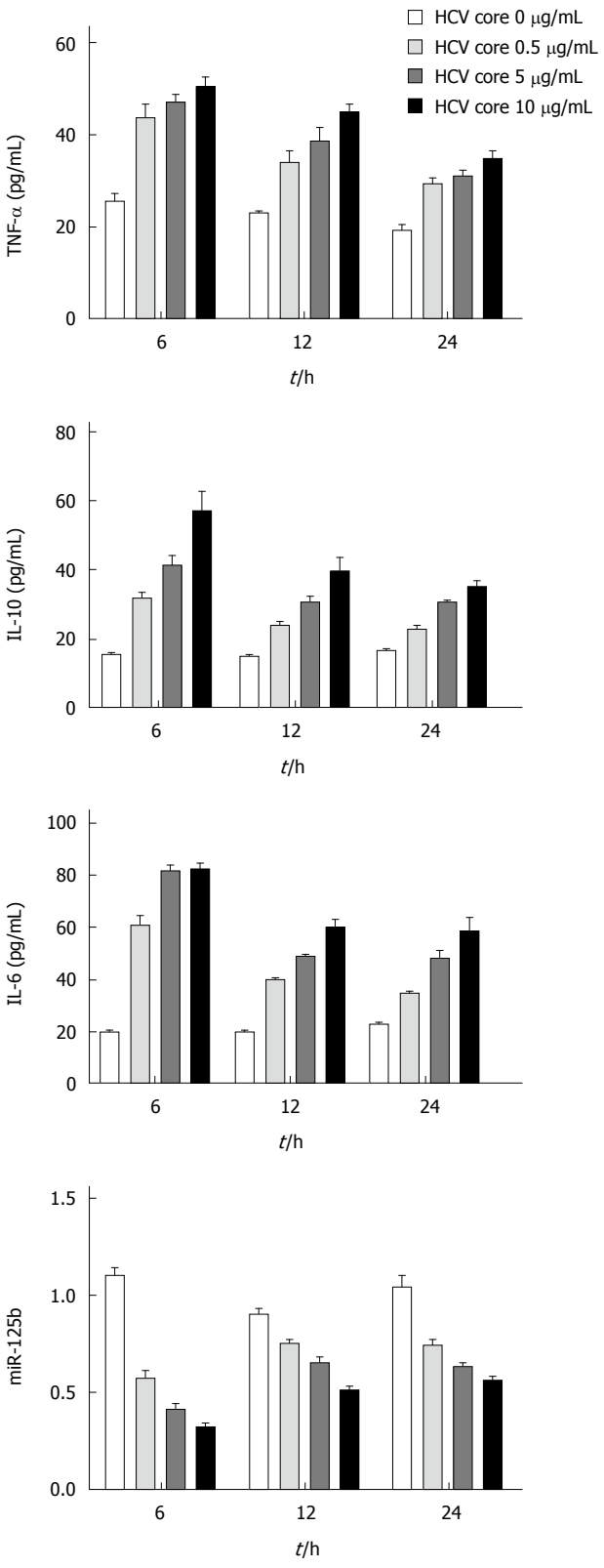

Figure 1 Increased cytokine expression and decreased miR-125b expression in hepatitis C virus core protein treated THP-1 cells. THP-1 cells were treated with HCV core recombinant protein at a final concentration of 0.5, 1.0 or 5.0 μg/mL and incubated for 6, 12 or 24 h. The levels of tumor necrosis factor (TNF)-α, interleukin (IL)-10, and IL-6 in culture supernatants were analyzed by enzyme-linked immunosorbent assay. The cellular miR-125b levels were determined by real-time quantitative polymerase chain reaction. The data are representative of three experiments and shown as mean ± SEM.

incubated with various concentrations (0.5, 1, 5 μg/mL) of HCV core protein for different time periods. Expression of miR-125b, miR-146 and miR-155, along with selected cytokine genes (TNF-α, IL-6 and IL-10) was examined by RT-qPCR analyses. Consistent with those of Yao *et al*[17], our results (Figure 1) showed that HCV core protein could directly stimulate THP-1 cells to express TNF-α, IL-6 and IL-10. The induction was dose-dependent; HCV core protein at 0.5 μg/mL significantly induced these cytokines more than two folds. The induction was also rapid; it peaked at 6 h after HCV core protein stimulation and slowly leveled off through the study period (24 h).

In parallel to the cytokine induction, miR-146 and miR-155 expression was also induced (data not shown)[12]. In contrast, miR-125 expression was down-regulated by HCV core stimulation in a dose-dependent manner. The miR-125b expression reached the lowest level (decreased by 68%) at 6 h with 5.0 μg/mL HCV core protein challenge. An inverse correlation between miR-125b expression and that of cytokines suggests that miR-125b may play a regulatory role in HCV-induced cellular responses.

TLR2 or MyD88 genes knockdown abolishes the suppressive effect of HCV core protein on miR-125b expression

TLR2 and its downstream signaling protein Myd88 play a prominent role in HCV core-induced inflammatory responses[18]. We employed a gene knockdown strategy to test if TLR2 or Myd88 could be directly involved in the repression of miR-125b. THP-1 cells were transfected with siRNA plasmids that could silence TLR2, MyD88 or the control plasmid for 24 h. The transfected cells were challenged with HCV core protein at 5 μg/mL for an additional 6 h. The miR-125b level was detected by RT-qPCR analysis. As shown in Figure 2A and 2B, we found that TLR2 and MyD88 mRNA and protein were reduced more than 2.5-fold when cells were transfected with these siRNA plasmids. When these transfected cells were exposed to HCV core protein, miR-125b expression was significantly restored compared to that of control-transfected cells. ($P < 0.05$, $n = 3$, Figure 2C). This result suggests that the suppression of miR-125b expression by HCV core treatment depends at least in part on the TLR2/MyD88 signaling.

Upregulation of cytokines by HCV core protein is abrogated by the miR-125b mimic

The inverse correlation noted between cytokine and miR-125b in HCV core protein-treated cells prompts us to examine whether miR-125b can directly participate in regulation of these HCV core protein-induced events. We transfected THP-1 cells with a chemically modified miR-125bM (miR-125b mimic) RNA oligos or the control RNA oligos for 24 h, followed by HCV core

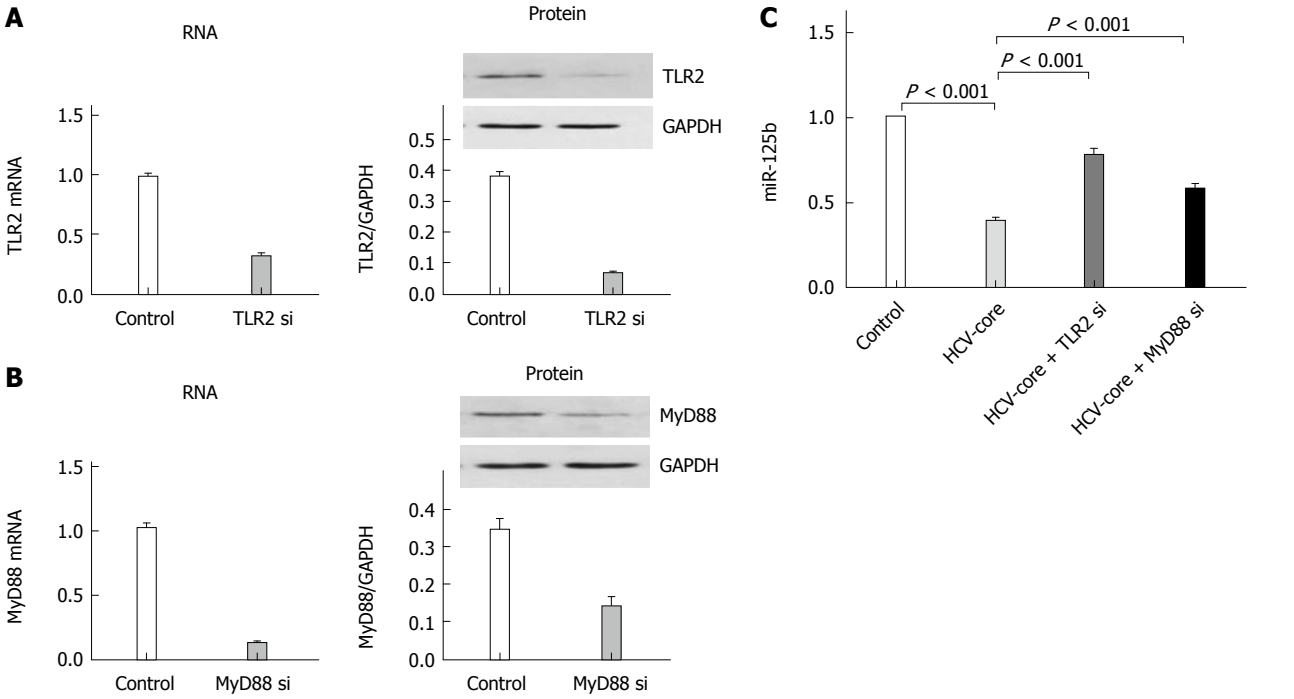

Figure 2 TLR2-MyD88 pathway dependent HCV core protein-induced miR-125b repression. TLR2 (A) or MyD88 (B) mRNA was analyzed by real-time quantitative polymerase chain reaction (RT-qPCR) and protein lysates were analyzed by Western blot at 6 h post transfection. The transfected cells were infected with HCV core protein at 5 μg/mL for 6 h and miR-125b expression was detected by RT-qPCR analysis (C). Cell data are representative of three experiments and are shown as mean ± SEM ($P < 0.001$ by unpaired two-tailed Student's *t*-test).

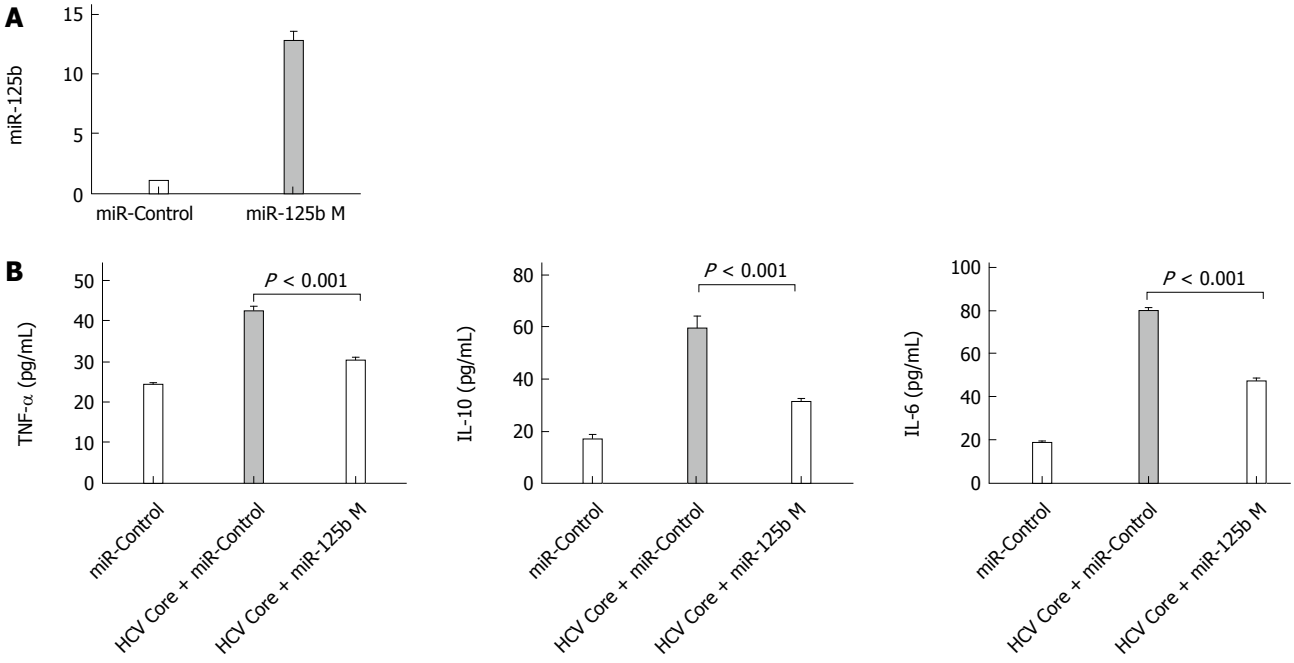

Figure 3 The miR-125b mimic abrogats hepatitis C virus core protein-induced upregulation of cytokines. A: Real-time quantitative polymerase chain reaction analysis of miR-125b levels in THP-1 cells transfected with 0.4 nmol/L miRNA negative control (miR-control) or miR-125b mimic (miR-125b M), respectively; B: Twenty four hours after transfection, cells were treated with 5.0 μg/mL HCV core protein for an additional 6 h. The concentrations of tumor necrosis factor (TNF)-α, interleukin (IL)-10 and IL-6 in culture supernatants were analyzed by enzyme-linked immunosorbent assay. These data are representative of three experiments and shown as mean ± SEM ($P < 0.001$ by unpaired two-tailed Student's *t*-test).

stimulation at 5.0 μg/mL for 6 h and examined the expression of TNF-α, IL-6 and IL-10. The effectiveness of miR-125b mimic transfection is shown in Figure 3A. In the experiment in which cells were treated with HCV core protein (Figure 3B), we found that transfection with control miRNA had no effect on the enhancement of cytokine expression by HCV core stimulation but that transfection with miR-125b mimic abrogated

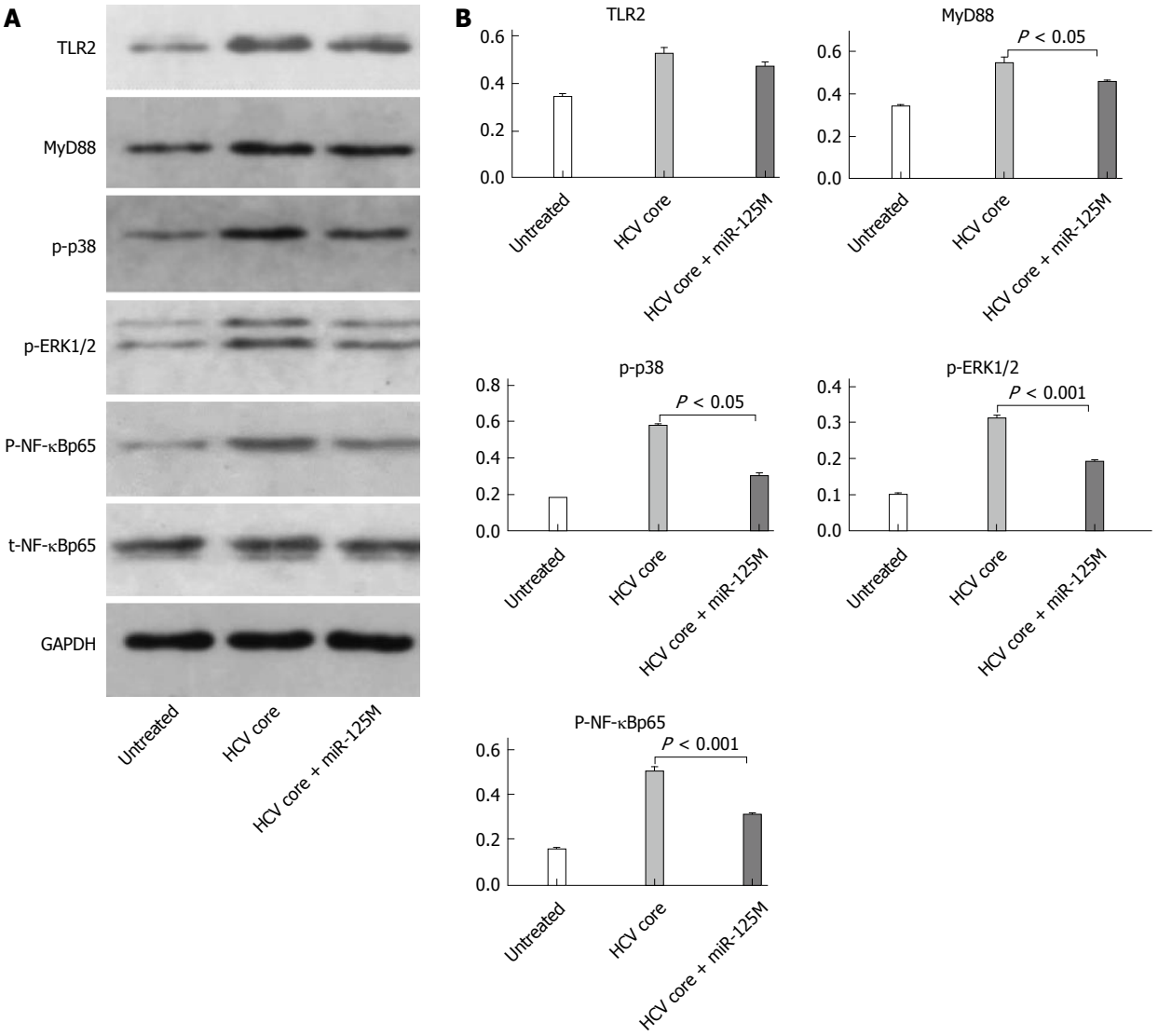

Figure 4 MiR-125b suppresses phosphorylation of NF-κBp65, ERK1/2 and p38. A: THP-1 cells were transfected with control or miR-125b mimic (miR-125bM) for 24 h, followed by incubation with hepatitis C virus (HCV) core protein at 5.0 μg/mL for 6 h. Western blot analysis of Toll-like receptor 2 (TLR2), MyD88, NF-κB, phosphorylated NF-κB, phosphorylated ERK1/2 and phosphorylated P38 in the collected cells; B: Densitometric quantification of the western blot data was performed using Quantity One Software. These data are representative of three experiments and shown as mean ± SEM ($P < 0.05$, $P < 0.001$ by unpaired two-tailed Student's *t*-test).

upregulated expression of TNF-α, IL-6 and IL-10 by 66%, 54% and 66%, respectively, compared with the control. These results indicate that overexpression of miR-125b down-regulates HCV-mediated cytokine induction.

MiR-125b mimic suppresses TLR2/MyD88 signaling

To understand the mechanism of miR-125b in regulating cytokine expression in HCV core protein-treated cells, we performed immunoblot analyses to determine the expression levels of the signaling molecules possibly involved in the TLR signaling pathways. THP-1 cells were transfected with or without miR-125b mimic and incubated with 5 μg/mL HCV core protein. The expression of various TLR signaling proteins was analyzed. As shown in Figure 4A, expression of TLR2 and MyD88 and the phosphorylation of their downstream signaling proteins ERK1/2, NF-κBp5 and p38 were up-regulated by HCV core treatments. The transfection of cells with the miR-125b mimic did not change the expression of TLR2 or NF-κBp65 but slightly down-regulated that of MyD88. In contrast, the phosphorylation of NF-κBp65, p38 and ERK1/2 was significantly down-regulated. Quantitation of three independent experiments showed two-fold down-regulation (Figure 4B).

DISCUSSION

In this study, we used recombinant HCV core protein-treated THP-1 cells as a model to investigate the interaction between HCV and monocytes. HCV core protein has been shown to be capable of directly interacting with various cellular proteins including

TLR2 and resulting in activation of TLR2-MyD88 signaling cascade in monocytes[18]. In agreement with these findings, our current findings indicate that HCV core protein can induce a similar profile of cytokine production in THP-1 cells. We further extended the study by demonstrating for the first time that treatment with HCV core protein suppressed the expression of miR-125b in a dose- and time-dependent manner (Figure 1). In addition, we demonstrated that this suppression was dependent on the TLR2/MyD88 pathway (Figure 2). However, the mechanism by which miR-125b expression is inhibited by HCV core protein treatment remains unknown.

MiR-125b has gained special interest in the field of cancer research because of its dysregulation in a broad variety of tumors[19]. Studing its role in tumorigenesis suggests that miR-125b could have the opposite effect depending on cellular context; miR-125b is upregulated in some tumors such as colon cancer and hematopoietic tumors, suggesting its oncogenic potential capability of facilitating cell proliferation and blocking apoptotic pathways[20,21]. On the other hand, miR-125b is down-regulated in other types of tumors, such as mammary tumors and HCC. The overexpression of miR-125b inhibits HCC cell proliferation *via* promoting apoptosis and other anti-tumor mechanisms[22].

Our finding that forced expression of miR-125b suppressed HCV core protein-induced cytokine production (Figure 3) and phosphorylation of the TLR/MyD88 signaling cascade (Figure 4) supports the notion that miR-125b suppresses HCV core protein-mediated events by inhibition of TLR2/MyD88 signaling. Although many receptors, signaling molecules, and transcriptional factors of the TLR signaling pathways have been verified to be regulated by various miRNAs[23], the molecules targeted by miR-125b in the TLR2/MyD88 signaling pathway remain unknown. Like other microRNAs, it is assumed that miR-125b exerts its biological function by directly targeting the 3′ untranslated (3′UTR) region of genes. Target site predictions using web-accessible miRNA database search programs (http://www.microrna.org,http://www.miRBase.org and http://www.targetscan.org) identified a miR-125b target site within the 1.3 kB 3′UTR of murine MyD88 mRNA. A study by Wang *et al*[24] indicated that the treatment of mouse macrophages with a miR-125b inhibitor induced MyD88 expression and that cellular transfection with a murine miR-125b mimic down-regulated the reporter gene activities mediated by 3′UTR MyD88 construct. These results provided clear evidence that murine miR-125b suppresses TLR/MyD88 by directly targeting MyD88 mRNA. We are currently testing this hypothesis by transfecting HCV core protein-treated THP-1 cells with the 3′UTR of MyD88 reporter construct.

In summary, our findings indicated that miR-125b may function as a negative regulator for HCV core protein-induced cellular events, which may provide insight into the role of miR-125b in the innate immune responses against HCV in monocytes. Our study may lay some groundwork for the development of a novel therapeutic approach for treating HCV core protein-induced inflammation and immune activation.

COMMENTS

Background

Macrophages/monocytes have been shown to be important immune cells mediating both innate and adaptive immunity in hepatitis C virus (HCV)-infected patients. Many anti-pathogen pathways, including pattern recognition Toll-like receptors (TLRs) mediating signaling, are known to be regulated by a network of microRNAs. MiR-125b is highly expressed in peripheral blood mononuclear cells, particularly monocytes/macrophages; however, its role in regulating monocyte immune responses induced by HCV has not yet been investigated.

Research frontiers

Recent studies have shown that several microRNAs, including miR-21, miR-146a and miR-155, are involved in regulation of virus-host interactions and may play important roles in the pathogenesis of HCV infection.

Innovations and breakthroughs

This is the first study to report that miR-125b may negatively regulate HCV core protein-induced host immune responses by targeting TLR2/MyD88 signaling in monocytes.

Applications

The present findings indicated that miR-125b may function as a negative regulator for HCV induced cellular events, which may provide insight into the role of miR-125b in the innate immune responses against HCV in monocytes. This study may lay some groundwork for the development of a novel therapeutic approach for treating HCV-induced inflammation and immune activation.

Terminology

MicroRNAs are highly conserved endogenous non-coding small RNAs that act as translational repressors to regulate a wide range of biological processes. They have also been implicated in the pathogenesis, diagnosis, and therapeutic aspects of HCV infections. miR-125b is highly expressed in peripheral blood mononuclear cells, particularly in monocytes/macrophages, and its expression is inversely associated with better treatment outcomes for chronic HCV infection.

Peer-review

The authors investigated the possible role of miR-125b in regulating monocyte immune responses induced by HCV core protein. This study found that cytokine production was up-regulated and miR-125b expression was down-regulated by HCV core protein treatment through TLR2/MyD88 signaling in THP-1 cells. Overexpression of miR125b resulted in suppressed MAPK and NF-κB activity and cytokine production. This research is novel and manuscript presentation and readability are good. Additionally, the manuscript is well organized and its results are sound with respect to the integrity of the paper.

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ORIGINAL ARTICLE

Case Control Study

Non-alcoholic fatty liver disease is not associated with a lower health perception

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Author contributions: Zelber-Sagi S conceived and designed the study; Schlesinger D and Zelber-Sagi S analyzed the data; Lotan R, Webb M and Zelber-Sagi S performed the data collection; Halpern Z, Santo E and Shibolet O conducted on data collection; Mlynarsky L, Shibolet O and Zelber-Sagi S wrote the manuscript; all authors critically reviewed the manuscript and approved it.

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Informed consent statement: All participants signed an informed consent.

Conflict-of-interest statement: The authors declare no conflicts of interest.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at zelbersagi@bezeqint.net.

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Abstract

AIM: To examine the association between non-alcoholic fatty liver disease (NAFLD) and general health perception.

METHODS: This cross sectional and prospective follow-up study was performed on a cohort of a sub-sample of the first Israeli national health and nutrition examination survey, with no secondary liver disease or history of alcohol abuse. On the first survey, in 2003-2004, 349 participants were included. In 2009-2010 participants from the baseline survey were invited to participate in a follow-up survey. On both baseline and follow-up surveys the data collected included: self-reported general health perception, physical activity habits, frequency of physician's visits, fatigue impact scale and abdominal ultrasound. Fatty liver was diagnosed by abdominal ultrasonography using standardized criteria and the ratio between the median brightness level of the liver and the right kidney was calculated to determine the Hepato-Renal Index.

RESULTS: Out of 349 eligible participants in the first survey, 213 volunteers participated in the follow-up

cohort and were included in the current analysis, NAFLD was diagnosed in 70/213 (32.9%). The prevalence of "very good" self-reported health perception was lower among participants diagnosed with NAFLD compared to those without NAFLD. However, adjustment for BMI attenuated the association (OR = 0.73, 95%CI: 0.36-1.50, *P* = 0.392). Similar results were observed for the hepato-renal index; it was inversely associated with "very good" health perception but adjustment for BMI attenuated the association. In a full model of multivariate analysis, that included all potential predictors for health perception, NAFLD was not associated with the self-reported general health perception (OR = 0.86, 95%CI: 0.40-1.86, *P* = 0.704). The odds for "very good" self-reported general health perception (compared to "else") increased among men (OR = 2.42, 95%CI: 1.26-4.66, *P* = 0.008) and those with higher performance of leisure time physical activity (OR = 1.01, 95%CI: 1.00-1.01, *P* < 0.001, per every minute/week) and decreased with increasing level of BMI (OR = 0.91, 95%CI: 0.84-0.99, *P* = 0.028, per every kg/m^2) and older age (OR = 0.96, 95%CI: 0.93-0.99, *P* = 0.033, per one year). Current smoking was not associated with health perception (OR = 1.31, 95%CI: 0.54-3.16, *P* = 0.552). Newly diagnosed (naive) and previously diagnosed (at the first survey, not naive) NAFLD patients did not differ in their self-health perception. The presence of NAFLD at the first survey as compared to normal liver did not predict health perception deterioration at the 7 years follow-up. In terms of health-services utilization, subjects diagnosed with NAFLD had a similar number of physician's visits (general physicians and specialty consultants) as in the normal liver group. Parameters in the fatigue impact scale were equivalent between the NAFLD and the normal liver groups.

CONCLUSION: Fatty liver without clinically significant liver disease does not have independent impact on self-health perception.

Key words: Non-alcoholic fatty liver disease; Health perception; Quality of life; Fatigue; Health-care services utilization

Core tip: In recent years there is overwhelming evidence that non-alcoholic fatty liver disease (NAFLD) is a major public health concern; the most common chronic liver disorder globally and associated with hepatic and extrahepatic morbidity and mortality. However, this study demonstrates that NAFLD diagnosis among a general population is not independently associated with lower general health perception nor is it associated with higher health care utilization. Moreover, NAFLD does not seem to predict health perception deterioration over the years. These findings imply that in the general population, NAFLD is not considered a disease in the eyes of the NAFLD beholder, probably until an advanced stage.

Mlynarsky L, Schlesinger D, Lotan R, Webb M, Halpern Z, Santo E, Shibolet O, Zelber-Sagi S. Non-alcoholic fatty liver disease is not associated with a lower health perception. *World J Gastroenterol* 2016; 22(17): 4362-4372 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i17/4362.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i17.4362

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is defined as fat accumulation in the liver, in the absence of significant alcohol intake. NAFLD is the most common chronic liver disorder globally[1], with a worldwide prevalence estimated from 6.3% to 33% and a median of 20%[2]. Recently, the prevalence of NAFLD was shown to be increasing in developing countries due to adoption of a Western lifestyle, the estimated prevalence of NAFLD varies from 20%-30% in Western countries to 5%-18% in Asia[3].

NAFLD is associated with hepatic and extrahepatic morbidity. Moreover, patients with NAFLD have reduced survival compared with the general population, primarily due to cardiovascular disease followed by malignancy[4-7]. In a Swedish cohort of NAFLD patients with a median follow-up of 27 years, 25% were diagnosed with cirrhosis and 14% with hepatocellular cancer[8]. NAFLD patients do not usually present with symptoms directly attributable to their underlying liver disease[9]. However, some patients report non-specific symptoms, including fatigue or malaise, daytime sleepiness and discomfort in the right upper abdominal quadrant[10]. Fatigue is the most common symptom in NAFLD patients and leads to impaired quality of life[11]. Lifestyle modification is the only established treatment in NAFLD, nevertheless, patients have low level of readiness for change and motivation to adopt a healthier lifestyle (particularly in the area of physical activity). Furthermore, it was shown that the severity of liver disease or liver enzymes elevation, have almost no impact on motivation to change[12].

To date, only a few studies examined quality of life parameters[13,14] or evaluated the utilization of health-care services among NAFLD patients[11,15]. Moreover, self-rated general health perception, a frequently assessed parameter in epidemiological research[16] and a powerful predictor for morbidity and mortality[17], has not been tested in NAFLD patients. Therefore, the current study was aimed to examine the association between NAFLD and general health perception along with fatigue and utilization of health-care services in a sample of a general population screened for NAFLD.

MATERIALS AND METHODS

Study design and population

This cross sectional and prospective follow-up study was performed on a cohort of a sub-sample of the first Israeli national health and nutrition examination survey (the MABAT Survey)[18]. On the first survey, MABAT LIVER study, 2003-2004, 349 participants were included. In 2009-2010 participants from the baseline survey were invited to participate in a follow-up survey. No difference was observed between subjects that participated in the follow-up study compared to those who did not participate in any demographic, anthropometric or biochemical parameters as previously reported[19]. In both surveys, individuals with any of the following were excluded from the study: presence of HBsAg or anti-HCV antibodies, fatty liver suspected to be secondary to hepatotoxic drugs, inflammatory bowel disease, celiac disease and excessive alcohol consumption ($\geqslant$ 30 g/d in men or $\geqslant$ 20 g/d in women)[2,20].

Data collection

On both baseline and follow-up surveys the data collected included: measurements of weight, height, and waist circumference following a uniform protocol, interview, biochemical tests, and ultrasound for the diagnosis of NAFLD, all performed on the same day at the Gastroenterology department of the Tel-Aviv Medical Center. All blood samples were drawn at the morning hours after a fast of at least 12 h and assessed by the same laboratory of the Tel-Aviv Medical Center.

A face-to-face interview was carried out in both surveys using a structured questionnaire, assembled by the Ministry of Health and used in national surveys[18], that included demographic details, questions on health status, self-reported general health perception, alcohol consumption, smoking and physical activity habits, frequency of physician's visits and hospitalization. To avoid report bias, the participants were informed on their US and blood tests results only after filling in the questionnaires.

Fatigue was assessed by the fatigue impact scale (FIS)[21], including 7 questions regarding alertness, decreased work volume, less motivation for physical effort, difficulties in decision making or in thinking process and decreased activity[21]. The fatigue score represents the sum of questions with a positive response. Fatigue as a reason for physical inactivity was assessed by multi-choice question that evaluated the reasons for physical inactivity.

Self-reported general health perception was estimated with one simple question that was highly validated as an indication to general health status and is commonly used in surveys worldwide[17,22,23]. The question was "what is your general health status?" and the answers were: 1- "very good", 2- "good" 3- "not so good" 4- "poor".

Utilization of health service was estimated by a questionnaire assembled by the Israeli Center for Disease Control and used in the Israeli National Health Interview Survey[24]. Numbers of physician's visits (general physicians and specialty consultants) and hospitalization were assessed by a series of multi-choice questions.

Fatty liver was diagnosed by abdominal ultrasonography using standardized criteria[25]. Ultrasonography was performed in all subjects both at baseline and at follow up with the same equipment (EUB-8500 scanner Hitachi Medical Corporation, Tokyo, Japan) and by the same experienced radiologist (Webb M) as previously described[26-28]. The radiologist was blinded to the laboratory values and medical history of the participants. During the ultrasonography, a histogram of brightness levels, *i.e.*, a graphical representation of echo intensity within a region of interest was obtained. The ratio between the median brightness level of the liver and the right kidney was calculated to determine the Hepato-Renal Index (HRI). The HRI has been previously demonstrated to be highly reproducible and was validated against liver biopsy[29].

The study was approved by the institutional review board of Tel Aviv medical center and all participants signed an informed consent.

Statistical analysis

All statistical analyses were performed using SPSS version 22.0 for Windows (SPSS Inc., Chicago, IL, United States). Continuous variables are presented as mean ± SD, while categorical variables are presented in percentage. Univariate analyses were used for the comparison of variable's distribution between the study groups. To test differences in continuous variables between two groups the independent samples *t*-test (for normally distributed variables) or the Mann-Whitney *U* test (if non-parametric tests were required) were performed. To test differences in continuous variables between more than two groups the One-Way ANOVA was performed. To test the differences in categorical variables the Pearson χ^2 test was performed. The evaluation of the association between NAFLD and the prevalence of "very good" health perception, adjusting for potential confounders, was performed with multivariate logistic regression analysis, presenting odds ratio (OR) and confidence intervals (CI). The potential confounders included in the multivariate model were: gender, age, body mass index (BMI) and behavioral factors: current smoking and duration of performance of leisure time physical activity in the past year. $P < 0.05$ was considered statistically significant for all analyses. The statistical methods of this study were reviewed by Dr. Shira Zelber-Sagi, RD, PhD. Head of nutrition and behavior program, School of Public Health, the University of Haifa and Tel-Aviv Medical Center.

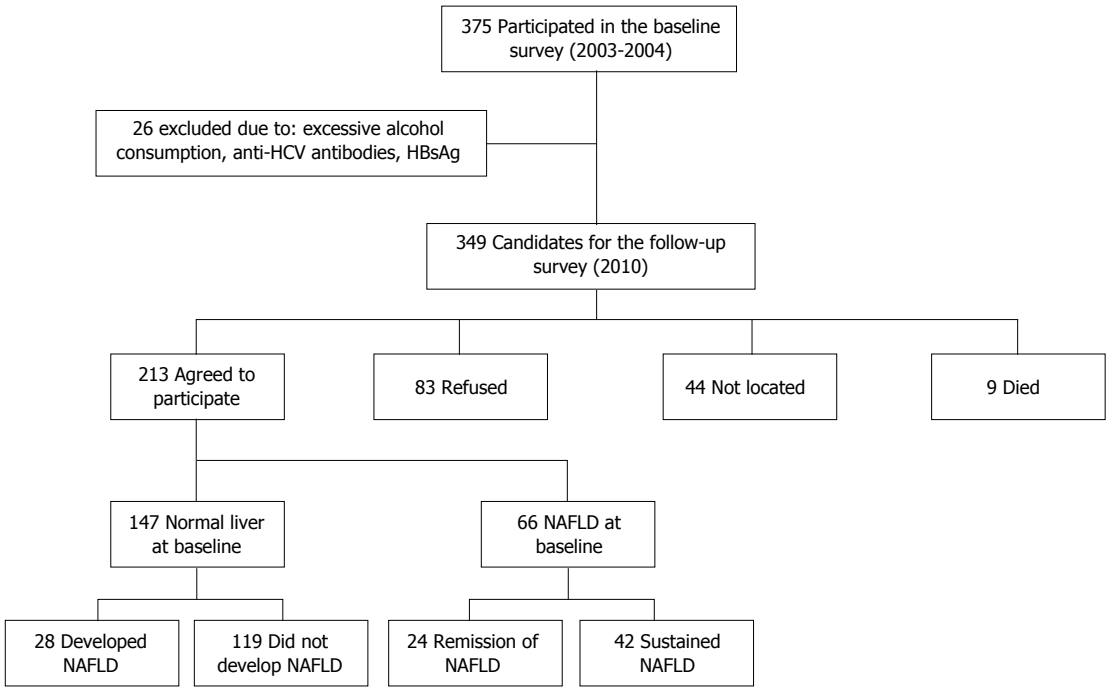

Figure 1 Flow chart of the study population. NAFLD: Non-alcoholic fatty liver disease.

Table 1 Comparison between non-alcoholic fatty liver disease and normal liver groups in the follow-up survey (mean ± SD, unless otherwise stated)

Parameter (normal range)	Entire cohort (n = 213)	NAFLD (n = 70)	Normal liver (n = 143)	P value
Age (yr)	57.96 ± 9.58	57.87 ± 8.12	58.01 ± 10.25	0.917
Gender (% males)	54.0	58.6	51.7	0.348
BMI (kg/m^2) (20-25)	28.15 ± 4.59	31.20 ± 4.38	26.66 ± 3.92	< 0.001
Waist circumference men (< 102 cm)	94.21 ± 10.47	98.88 ± 9.01	91.66 ± 10.40	< 0.001
Waist circumference women (< 88 cm)	83.30 ± 11.01	93.49 ± 9.50	79.02 ± 8.55	< 0.001
Current smoker (%)	17.4	25.7	13.3	0.025
Leisure time physical activity (min/wk)	96.17 ± 144.97	63.00 ± 107.92	113.12 ± 158.32	0.008
Years of education (%)				0.333
< 12	43.4	38.0	45.9	
12	17.6	24.0	14.7	
> 12	39.0	38.0	39.4	
ALT (U/L) (5-39)	24.01 ± 8.60	28.31 ± 9.07	21.91 ± 7.53	< 0.001
Glucose (mg/dL) (70-110)	93.62 ± 25.02	101.21 ± 25.62	89.90 ± 23.95	0.002
Insulin (U/mL) (5-25)	20.39 ± 9.55	24.67 ± 11.22	18.33 ± 7.89	< 0.001
HbA1C (%) (3.9-6.0)	5.94 ± 0.75	6.27 ± 0.78	5.78 ± 0.68	< 0.001
Cholesterol (mg/dL) (150-200)	184.45 ± 34.91	185.51 ± 38.34	183.93 ± 33.24	0.757
Triglycerides (mg/dL) (50-175)	109.27 ± 55.39	133.36 ± 55.01	97.48 ± 51.81	< 0.001
Hepatorenal index	1.30 ± 0.35	1.70 ± 0.31	1.09 ± 0.10	< 0.001

NAFLD: Non-alcoholic fatty liver disease; BMI: Body mass index; ALT: Alanine aminotransferase; HbA1C: Glycosylated hemoglobin.

RESULTS

Characteristics of the study population and comparison between NAFLD and normal liver groups

Out of 349 eligible participants in the first survey, 213 volunteers participated in the follow-up cohort and were included in the current analysis (Figure 1), 54% were men, mean age was 57.96 ± 9.58 years and mean BMI was 28.15 ± 4.59 kg/m^2. According to abdominal US, NAFLD was diagnosed in 70/213 (32.9%) participants on the follow-up survey. There were no significant age and gender differences between subjects with and without NAFLD. BMI, waist circumference (women and men), serum ALT, blood glucose, serum insulin, HbA1C and triglyceride levels were all significantly higher in the NAFLD group (Table 1).

NAFLD and self- reported general health perception

Among the normal liver group, 46.9% reported their general health perception status as "very good" *vs* 31.4% of the subjects in the NAFLD group (P = 0.032). However, stratification by BMI indicating a clinically significant overweight and above (BMI > 27 kg/m^2)[30]

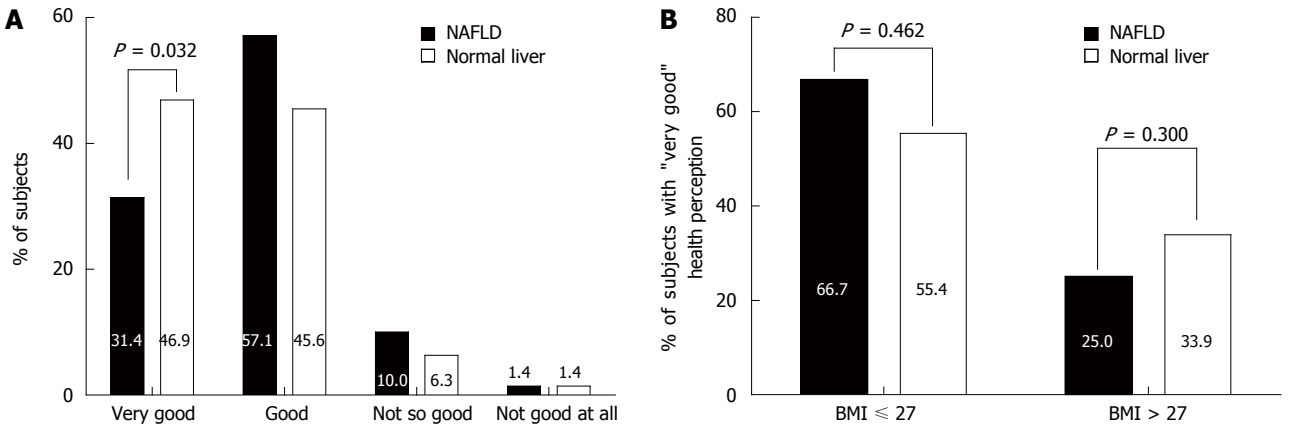

Figure 2 Distribution of self-rated health perception among non-alcoholic fatty liver disease and normal liver groups among the entire population (A), subjects with body mass index 27 and below (*n* = 95) and subjects with body mass index above 27 (*n* = 112) (B). *P* values represent the difference between NAFLD and normal liver groups in the distribution of "very good" health perception versus "else"- all other categories combined (Chi-square). NAFLD: Non-alcoholic fatty liver disease; BMI: Body mass index.

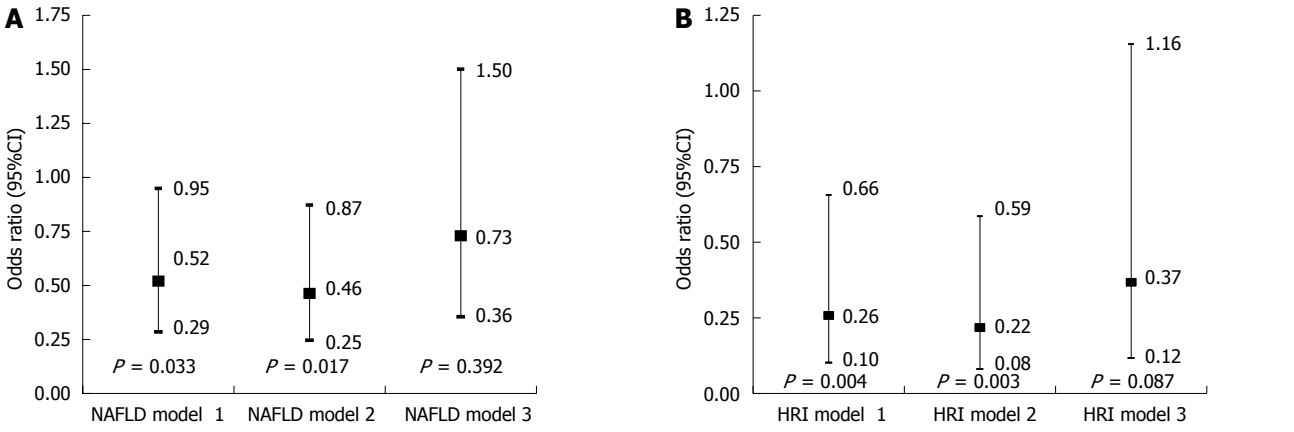

Figure 3 Univariate and multivariate association between non-alcoholic fatty liver disease and "very good" health perception (A) by non-alcoholic fatty liver disease diagnosed with regular US compared to normal liver (B) by hepato-renal index level (per one unit increase in the index) [odds ratio (95%CI)]. Model 1: Crude; Model 2: Adjusted for age and gender; Model 3: Adjusted for age, gender and BMI; NAFLD: Non-alcoholic fatty liver disease; BMI: Body mass index; HRI: Hepato-renal index.

eliminated this difference (Figure 2).

In a univariate analysis, the presence of NAFLD was associated with lower odds for a "very good" health perception (compared to "else") (OR = 0.52, 95%CI: 0.29-0.95, *P* = 0.033). This negative association remained significant with adjustment for age and gender (OR = 0.46, 95%CI: 0.25-0.87, *P* = 0.017). However, with farther adjustment for BMI the association with the presence of NAFLD was attenuated (OR = 0.73, 95%CI: 0.36-1.50, *P* = 0.392) (Figure 3). Similar results were observed for the hepato-renal index; it was inversely associated with "very good" health perception in the crude and in the age and gender adjusted model but not with further adjustment for BMI (Figure 3).

In a full model of multivariate analysis, that included all potential predictors for health perception (gender, age, BMI and behavioral factors: current smoking and duration of performance of leisure time physical activity in the past year), NAFLD was not associated with the self-reported general health perception (OR = 0.86, 95%CI: 0.40-1.86, *P* = 0.704). The odds for "very good" self-reported general health perception (compared to "else") increased among men (OR = 2.42, 95%CI: 1.26-4.66, *P* = 0.008) and those with higher performance of leisure time physical activity (OR = 1.01, 95%CI: 1.00-1.01, *P* < 0.001, per every minute/week) and decreased with increasing level of BMI (OR = 0.91, 95%CI: 0.84-0.99, P = 0.028, per every kg/m^2) and older age (OR = 0.96, 95%CI: 0.93-0.99, *P* = 0.033, per one year). Current smoking was not associated with health perception (OR = 1.31, 95%CI: 0.54-3.16, *P* = 0.552).

NAFLD dynamics and the association with health perception

All the participants in the study underwent two abdominal US evaluations with a 7 years interval. Forty-two (19.7%) participants were diagnosed with NAFLD in both US assessments, in 28 (13.1%) NAFLD

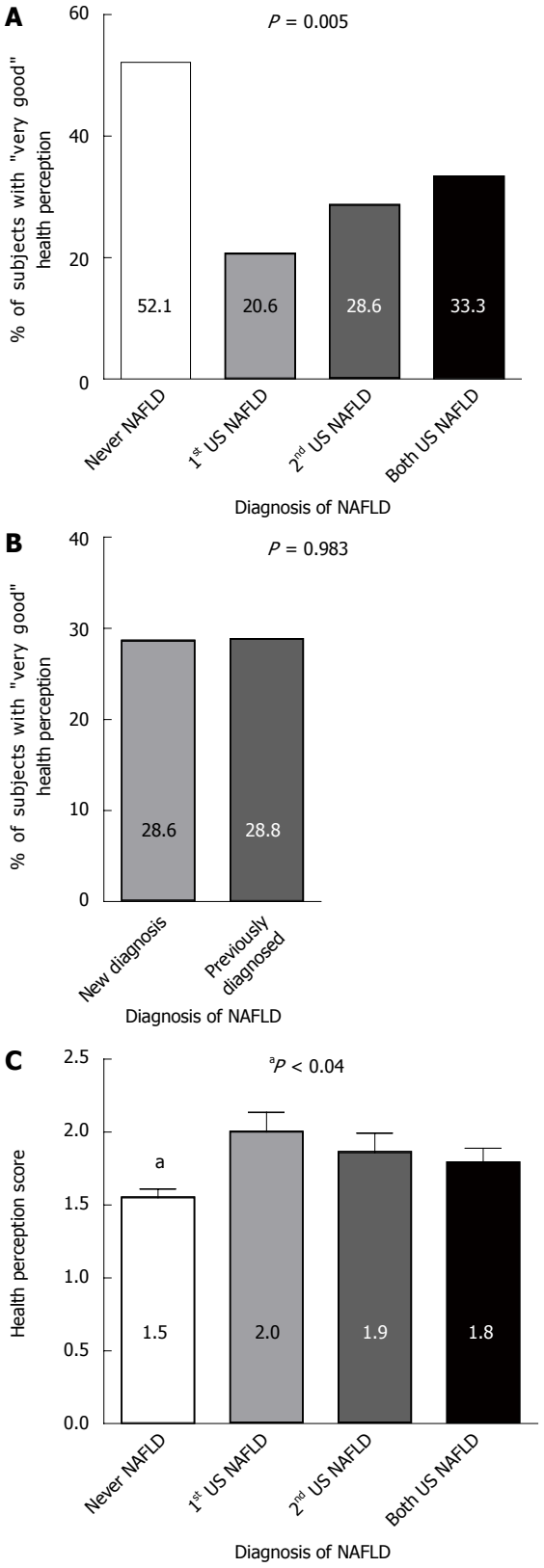

Figure 4 Health perception by past and present diagnosis of fatty liver on ultrasound (never NAFLD *n* = 119, 1st US NAFLD *n* = 24, 2nd US NAFLD *n* = 28, both US NAFLD *n* = 42). A: Percent of subjects with "very good" health perception by NAFLD dynamics; B: Percent of subjects with "very good" health perception by naive (newly diagnosed) and not naive (previously diagnosed) NAFLD status; C: The average score of health perception by NAFLD dynamics (the lower the score the higher the health perception; 1 = "very good", 4 = "not good at all") (a never NAFLD *vs* every other group). NAFLD: Non-alcoholic fatty liver disease.

was observed only in the latter US and in 24 (11.3%) NAFLD was diagnosed only in the first US but not in the second one, 119 (55.9%) participants had a normal liver in both US assessments.

The prevalence of "very good" health perception was higher among the subgroup that was never diagnosed with NAFLD (52.1%) compared to participates who were ever diagnosed with NAFLD during the study (only in the first evaluation, only in the second evaluation, or in both evaluations, 20.6%, 28.6% and 33.3%, respectively, P = 0.005), without significant difference between the "ever NAFLD" groups (Figure 4A). Furthermore, naive (newly diagnosed) and previously diagnosed NAFLD patients (who already knew they have NAFLD) did not differ in their prevalence of "very good" health perception (Figure 4B). Similarly, the average score of general health perception (1 = "very good", 4 = "poor") in patients who were never diagnosed with NAFLD was significantly lower than the score in patients "ever diagnosed" with NAFLD (P < 0.04 for all comparisons), but no significant differences were observed between all "ever diagnosed" with NAFLD groups (Figure 4C).

Health perception dynamics and the association with NAFLD

Among those diagnosed as normal liver at the first evaluation, 61/145 (42.1%) estimated their health as "very good" and among those with NAFLD at the first evaluation, 15/66 (22.7%) had "very good" health perception (P = 0.007). There was no difference between the NAFLD and the normal liver groups (as diagnosed at the first evaluation) in the dynamics of "very good" health perception between the first and follow-up surveys; 75.8% *vs* 70.3% retained, 9.1% *vs* 11.7% had a reduction and 15.2% *vs* 17.9% had improvement in health perception, respectively (P = 0.712).

Fatigue and avoidance from physical activity

The total time spent in all types of leisure time physical activity per week was twofold higher among subject without NAFLD as compared to those with NAFLD (113.12 ± 158.32 min/wk *vs* 63.00 ± 107.92 min/wk, P = 0.008).

In the entire cohort, 70/213 (32.9%) reported avoidance of physical activity. The main reason for avoidance was boredom (37.1%), followed by no available time (20.0%) and fatigue (12.9%). There was no significant difference between subjects with and without NAFLD in the main reason for avoidance from physical activity (P = 0.163). Only 2/26 (7.7%) in the NAFLD group *vs* 7/44 (15.9%) in the normal liver group reported fatigue as the main reason for avoidance from physical activity with no difference between groups (P = 0.321).

According to the FIS questionnaire, there was no significant difference between the NAFLD and the normal liver groups in any parameter of fatigue (data

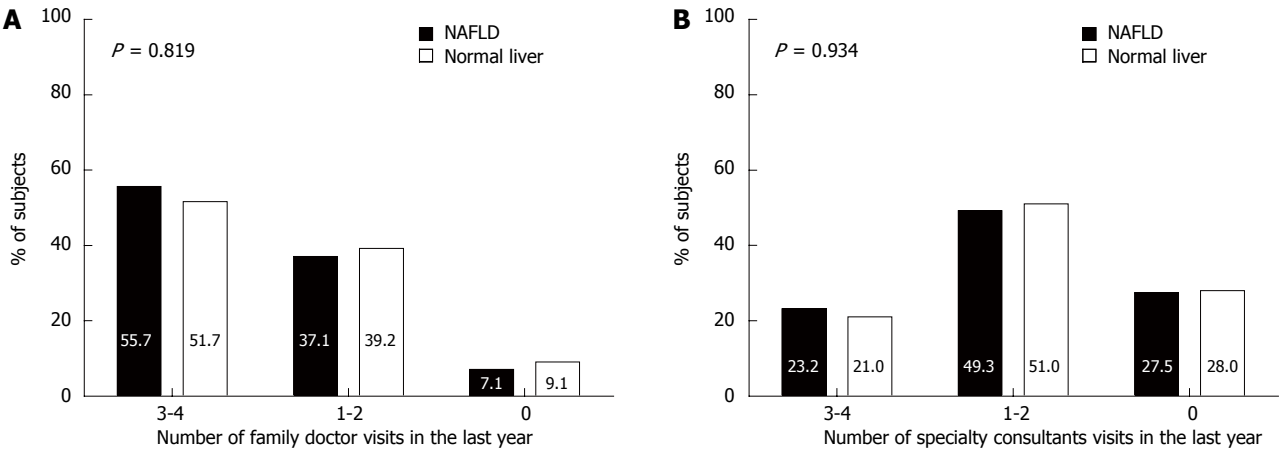

Figure 5 Comparison between non-alcoholic fatty liver disease and normal liver groups in the distribution of frequency of family doctor visits (A) and in the distribution of frequency of specialty consultants (B). NAFLD: Non-alcoholic fatty liver disease.

not shown, $P \geqslant 0.343$ for all) including the need to reduce daily activity due to fatigue (20.0% *vs* 19.6%, respectively, $P = 0.781$).

NAFLD and utilization of health-care services

There was no significant difference between subjects with and without NAFLD in the frequency of family doctor visits in the last year (Figure 5A). Furthermore, there was no difference between subjects with and without NAFLD in the frequency of specialty consultants in the last year (Figure 5B), in the occurrence of hospitalization in the last 5 years (28.6% *vs* 29.4%, respectively, $P = 0.904$) and in the number of hospitalization events (1.45 ± 0.99 *vs* 1.55 ± 1.47, respectively, $P = 0.789$).

DISCUSSION

NAFLD is emerging as a leading cause for chronic liver disease, cirrhosis and hepatocellular carcinoma[2], thus early diagnosis, life style modifications and treatment are essential. The association between NAFLD and general health perception is debatable. Perceived health status is a reflection of both physical and psychological self-perception and has a well-established association with adverse outcomes[31-33]. The question "How is your health in general?" is well validated and a good predictor of future health care utilization and mortality[34,35]. In this study, "very good" health perception was less prevalent among subject with NAFLD compared to subject with normal liver. However, controlling for BMI attenuated the association between both the presence of NAFLD and the amount of liver fat and self-reported health perception. We acknowledge that multicollinearity exists between NAFLD and obesity, the latter was associated with a lower health perception. However, we aimed to learn if NAFLD as a distinct entity is associated with a lower health perception and thus controlled for BMI. To do that, we not only controlled for BMI in a multivariate analysis, but also stratified on it and in both cases it attenuated the association between the presence of NAFLD and self-reported health perception. Moreover, deterioration of "very good" health perception with time could not be predicted by NAFLD. This finding indicates that despite the multiple negative health outcomes of NAFLD, patients don't feel or think of themselves as sick. This notion is also supported by other findings in our study. First, the NAFLD patients do not utilize more health services as measured by physician visits. Second, even though time spent in leisure time physical activity was lower among NAFLD subjects compared to normal liver controls, fatigue as an explanation for lack of physical activity was evenly reported between the groups. Furthermore, no difference in FIS was noted as well. Lastly, patients with a recent diagnosis of NAFLD (on the follow-up survey), who were unaware of the diagnosis at the time of the interview, had similar health perception as those with "long standing" NAFLD which was detected during the first survey, indicating that patients do not perceive NAFLD as a serious health threat.

Self-rated health has not been previously tested in NAFLD patients, but was tested in relation to diabetes mellitus indicating a greater chance for poor self-rated health among diabetic patients[36]. The difference between the perception of NAFLD and diabetes may stem from the "seniority" of diabetes in terms of disease recognition among physicians and public, the common use in medications in diabetes versus lack of medical treatment in NAFLD and additional diabetes-related complications related to a lower quality of life[37].

Significant positive predictors for "very good" health perception were male gender and regular performance of exercise and negative predictors were BMI and age. Similarly, according to the OECD cross-country comparisons of perceived good health status, in the vast majority of participating countries, men were more likely than women to report good health, and health perception tended to worsen with age[38].

As opposed to the scant data regarding health

perception, fatigue is more extensively investigated among NAFLD patients. In a cohort from Newcastle (United Kingdom) 44% of NAFLD patients experienced significant fatigue which was not correlated to thyroid function[39], insulin resistance or severity of liver disease[13]. Fatigue (assessed with FIS) among NAFLD patients was also significantly higher compared with age and sex matched controls[11,13]. The fatigue in NAFLD can be explained by lower blood pressure and autonomic dysfunction, but it may also be that relative hypotension is secondary to fatigue, reflecting the decreased amount of physical activity undertaken by patients who perceive themselves as fatigued[11,14]. Another explanation is excessive daytime sleepiness, the cardinal symptom of obstructive sleep apnea (OSA), commonly associated with obesity and NAFLD. OSA is well correlated with insulin resistance, but the correlation to NAFLD is debatable[40,41]. However, in a prospective cohort study, moderate to severe liver steatosis was associated with more severe obstructive sleep apnea. Continuous positive airway pressure (CPAP) therapy for 3 years partially reversed these changes in the majority of patients[42]. Our results may differ from other studies due to different study populations. In the current study the NAFLD subjects were sampled from the general population and not from a selected population of a liver clinic at a medical center.

If indeed fatigue is comparable among NAFLD and normal liver subjects, why do NAFLD subjects exercise less than normal liver subjects? Explanatory factors could be: reduced cardio-respiratory fitness, weight-related arthrosis, psychological factors and a tendency towards a sedentary lifestyle, all correlated with the metabolic syndrome[20, 43].

Only few studies evaluated the utilization of health services among NAFLD patients supporting the hypothesis that NAFLD patients have higher utilization of health services compared with the normal liver group[11,15,44]. In a 5-year population-based follow-up study in Germany, the presence of NAFLD, defined by both presence of a hyperechogenic pattern of the liver and elevated serum alanine aminotransferase (ALT) levels, was associated with a 26% increase of overall health care costs, after controlling for co-morbidities[45]. The current study results are inconsistent with the limited literature, perhaps since the NAFLD subjects in this study were sampled from the general population, most of them having liver enzymes within the normal range, thus it is very likely that their NAFLD is at a less progressive and symptomatic state compared with NAFLD patients referred for treatment at a medical center which have higher risk for having NASH.

In this study, the utilization of health services was not increased among the NAFLD diagnosed subjects. This finding combined with the equivalent health perception might point towards lack of awareness and understanding that NAFLD is in fact a progressive disease that requires a closer medical surveillance. The misperception of NAFLD as a non-significant disease may also be attributed to the way the health practitioners perceive NAFLD, perhaps not as a disease in itself with potentially severe outcomes, and as a consequence the information they provide to patients and their disease management. Several studies have demonstrated that hepatogastroenterologists[46], primary care practitioners[47] and hospital non-hepatologists specialists[48] do consider NAFLD as a disease and major health problem and follow NAFLD patients, but it is still unclear how firm is the message provided to the patients.

How can this obstacle to patient care be overcome? Health policy approach to improve the patient's perception and self-management of NAFLD may be an implementation of a "multidisciplinary team approach" in which patients will be followed by physicians, dietitians, psychologists and physical activity supervisors[49]. Furthermore, general practitioners and hepatologists treating NAFLD patients should provide information and refer the patients to appropriate resources about NAFLD implications and treatment and have training in behavioral therapy. Similarly to the treatment approach of other chronic diseases, healthcare providers need to talk with their NAFLD patients more about the broader picture of complications; hepatocellular cancer, increased risk of diabetes, heart attack or stroke, with the message that risk reduction is possible[50]. The 5 *A*'s model (ask, advise, assess, assist, and arrange) may be useful as a tool to assist clinicians advising NAFLD patients to modify their behavior, assessing their interest in doing so, assisting in their efforts to change, and arranging appropriate follow-up[51].

Our study has several limitations to consider. First, the diagnosis of NAFLD was established by using noninvasive methods of abdominal US and HRI and the histologic diagnosis of inflammation and fibrosis could not be obtained in a sample of the general population. However, with regard to the diagnosis of steatosis, using abdominal US is the most common and acceptable first-line screening procedure for NAFLD in clinical practice and in epidemiological studies[20,52,53].

Second, as mentioned above, the NAFLD subjects were sampled from the general population, thus our sample may not represent the more severe forms of the disease. Last, the utilization of health services was self-reported instead of objectively measured and thus prone to a report bias that may have weakened the observed associations.

In conclusion, NAFLD diagnosis among a general population is not independently associated with lower general health perception nor is it associated with higher health care utilization. Moreover, NAFLD does not seem to predict health perception deterioration over the years. These findings imply that in the general population, NAFLD is not considered a disease in the eyes of the NAFLD beholder, probably until the progressive state.

COMMENTS

Background

Non-alcoholic fatty liver disease (NAFLD) is defined as fat accumulation in the liver, in the absence of significant alcohol intake. It is the most common chronic liver disorder globally with significant hepatic and extrahepatic morbidity. Moreover, patients with NAFLD have reduced survival compared with the general population, primarily due to cardiovascular disease followed by malignancy.

Research frontiers

In recent years there is overwhelming evidence that NAFLD is a major public health concern. However, self-rated general health perception, a frequently assessed parameter in epidemiological research and a powerful predictor for morbidity and mortality, has not been tested in NAFLD patients.

Innovations and breakthroughs

This study demonstrates that NAFLD diagnosis among a general population is not independently associated with lower general health perception nor is it associated with higher health care utilization. Moreover, NAFLD does not seem to predict health perception deterioration over the years. These findings imply that in the general population, NAFLD is not considered a disease in the eyes of the NAFLD beholder, probably until the advanced stage.

Applications

More efforts should be directed to establish the acknowledgment of NAFLD as an independent clinical entity with a potentially progressive course. Such a firm and clear message from the treating physician to the patients may promote motivation and adherence to lifestyle changes and a wiser health care utilization for a closer medical surveillance.

Terminology

Self-reported general health perception was estimated with one simple question that was highly validated as an indication to general health status and is commonly used in surveys worldwide. The question was "what is your general health status?" and the answers were: 1- "very good", 2- "good" 3- "not so good" 4- "poor". Hepato-Renal Index (HRI) - during the ultrasonography, a histogram of brightness levels, *i.e.*, a graphical representation of echo intensity within a region of interest is obtained in the liver and in the right kidney. The brightness level for each organ is recorded and the ratio between the median brightness level of the liver and the right kidney cortex is calculated to determine the HRI.

Peer-review

This is a cross sectional study aimed at evaluating the self-rated general health perception in a cohort of 213 subjects form a health survey in Israel. The article is generally well-written and has scientific value, given the high prevalence of NAFLD and the potential implications of its findings in formulating health care policies.

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ORIGINAL ARTICLE

Retrospective Study

Significance of functional hepatic resection rate calculated using 3D CT/^{99m}Tc-galactosyl human serum albumin single-photon emission computed tomography fusion imaging

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Abstract

AIM: To evaluate the usefulness of the functional hepatic resection rate (FHRR) calculated using 3D computed tomography (CT)/^{99m}Tc-galactosyl-human serum albumin (GSA) single-photon emission computed tomography (SPECT) fusion imaging for surgical decision making.

METHODS: We enrolled 57 patients who underwent bi- or trisectionectomy at our institution between October 2013 and March 2015. Of these, 26 patients presented with hepatocellular carcinoma, 12 with hilar cholangiocarcinoma, six with intrahepatic cholangiocarcinoma, four with liver metastasis, and nine with other diseases. All patients preoperatively underwent three-phase dynamic multidetector CT and ^{99m}Tc-GSA scintigraphy. We compared the parenchymal hepatic resection rate (PHRR) with the FHRR, which was defined as the resection volume counts per total liver volume counts on 3D CT/^{99m}Tc-GSA SPECT fusion images.

RESULTS: In total, 50 patients underwent bisectionectomy and seven underwent trisectionectomy.

Biliary reconstruction was performed in 15 patients, including hepatopancreatoduodenectomy in two. FHRR and PHRR were 38.6 ± 19.9 and 44.5 ± 16.0, respectively; FHRR was strongly correlated with PHRR. The regression coefficient for FHRR on PHRR was 1.16 (P < 0.0001). The ratio of FHRR to PHRR for patients with preoperative therapies (transcatheter arterial chemoembolization, radiation, radiofrequency ablation, *etc*.), large tumors with a volume of > 1000 mL, and/or macroscopic vascular invasion was significantly smaller than that for patients without these factors (0.73 ± 0.19 *vs* 0.82 ± 0.18, P < 0.05). Postoperative hyperbilirubinemia was observed in six patients. Major morbidities (Clavien-Dindo grade ⩾ 3) occurred in 17 patients (29.8%). There was no case of surgery-related death.

CONCLUSION: Our results suggest that FHRR is an important deciding factor for major hepatectomy, because FHRR and PHRR may be discrepant owing to insufficient hepatic inflow and congestion in patients with preoperative therapies, macroscopic vascular invasion, and/or a tumor volume of > 1000 mL.

Key words: ^{99m}Tc-galactosyl human serum albumin; Single-photon emission computed tomography; Hepatectomy; Functional hepatic resection rate; Parenchymal hepatic resection rate

Core tip: We evaluated the usefulness of the functional hepatic resection rate (FHRR) calculated using 3D computed tomography (CT)/^{99m}Tc-galactosyl human serum albumin (GSA) single-photon emission computed tomography fusion imaging and found a strong correlation between FHRR and the parenchymal hepatic resection rate (PHRR). However, FHRR and PHRR were discrepant because of insufficient hepatic inflow and congestion in patients with preoperative therapies, macroscopic vascular invasion, or a tumor volume of > 1000 mL.

Tsuruga Y, Kamiyama T, Kamachi H, Shimada S, Wakayama K, Orimo T, Kakisaka T, Yokoo H, Taketomi A. Significance of functional hepatic resection rate calculated using 3D CT/99mTc-galactosyl human serum albumin single-photon emission computed tomography fusion imaging. *World J Gastroenterol* 2016; 22(17): 4373-4379 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i17/4373.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i17.4373

INTRODUCTION

The development of postoperative liver failure (PHLF) is a major cause of morbidity and mortality after hepatectomy[1]. The mortality rate after major hepatectomy is reported as 2.0% to 6.8%[1-3]. A smaller remnant liver volume and an impaired hepatic functional capacity increase the risk of PHLF[4]. Preoperative estimation of the hepatic functional reserve is important for major hepatectomy[5]. Although the future remnant hepatic volume can be calculated by computed tomography (CT) volumetry[6], precise estimation of the future liver remnant function is difficult.

The asialoglycoprotein receptor is only expressed on the sinusoidal surfaces of mammalian hepatocytes. ^{99m}Tc-galactosyl human serum albumin (^{99m}Tc-GSA) was developed as a liver scintigraphy agent that binds to the asialoglycoprotein receptor on hepatocytes[7]. ^{99m}Tc-GSA scintigraphy is frequently used for evaluating the hepatic functional reserve, and several parameters for quantitative determination of hepatic function such as the hepatic uptake ratio to the liver plus heart at 15 min (LHL15)[8] and the maximum removal rate of ^{99m}Tc-GSA (Rmax)[9] have been reported. ^{99m}Tc-GSA single-photon emission computed tomography (SPECT) can acquire the regional distribution of hepatic function[10]. However, the spatial resolution of ^{99m}Tc-GSA SPECT is low. Furthermore, it is difficult to precisely estimate the functional reserve of the future liver remnant in patients requiring complicated resection. CT/^{99m}Tc-GSA SPECT fusion imaging has been reported to enable the precise evaluation of hepatic function distribution, given the high spatial resolution provided by CT[11]. Several parameters using CT/^{99m}Tc-GSA SPECT fusion imaging such as the preoperative total amount of receptor in the future remnant liver (R0-remnant)[12], the liver uptake value corrected for body surface area ($LUV_{(BSA)}$)[13] and the uptake index (UI)[14] have been reported to be useful for preoperative estimation of remnant hepatic function, but they were considerably complex. We considered hepatic resection rate is more intuitively usable.

In the present study, we calculated the functional hepatic resection rate (FHRR) using 3D CT/^{99m}Tc-GSA SPECT fusion imaging for patients undergoing major hepatectomy and determined its usefulness by correlation with the parenchymal hepatic resection rate (PHRR).

MATERIALS AND METHODS

Between October 2013 and March 2015, 57 patients who underwent bi- or trisectionectomy at the Department of Gastroenterological Surgery I, Hokkaido University Hospital were enrolled in this study. The baseline characteristics of the patients are shown in Table 1. All patients preoperatively underwent three-phase dynamic multidetector CT and ^{99m}Tc-GSA scintigraphy. Preoperative portal vein embolization (PVE) is performed for patients with a PHRR of > 60% at our institution[12]. Accordingly, 13 patients with a mean PHRR of 62.1% ± 7.9% (range, 47.0%-71.3%)

Table 1 Baseline characteristics of patients (n = 57) n (%)

Age mean (range)	65.7 (34-82)
Men/women	33/24
HBsAg positivity	10 (1.6)
HCV positivity	7 (1.2)
Diagnosis	
HCC	26 (45.6)
Hilar cholangiocarcinoma	12 (21.1)
Intrahepatic cholangiocarcinoma	6 (10.5)
Metastatic tumor	4 (7.0)
Hemangioma	4 (7.0)
Others	5 (8.8)
Child-Pugh score (5/6/7/8)	44/8/3/2
Child-Pugh classification (A/B)	52/5
ICGR$_{15}$, mean (range)	10.8 (1.5-44.3)
LHL$_{15}$, mean (range)	0.920 (0.712-0.973)
Preoperative therapies	18 (31.6)
TACE	6 (10.5)
Radiation	5 (8.8)
RFA	3 (5.3)
Partial resection	3 (5.3)
Chemotherapy	1 (1.8)
Tumor size > 1000 mL	5 (8.8)
Portal vein thrombosis	8 (14.0)
Hepatic vein thrombosis	3 (5.3)
Preoperative biliary drainage	15 (26.3)
Preoperative portal vein embolization	13 (22.8)

HCC: Hepatocellular carcinoma; ICGR$_{15}$: Indocyanine green retention at 15 min; LHL$_{15}$: The hepatic uptake ratio to the liver plus heart at 15 min of ^{99m}Tc-galactosyl human serum albumin; TACE: Transcatheter arterial chemoembolization; RFA: Radiofrequency ablation.

underwent preoperative PVE.

Hepatectomy

An algorithm (Hokkaido University Algorithm) incorporating the indocyanine green retention at 15 min and remnant liver volume is generally used to determine the nature of sectionectomy required, *e.g.*, bisectionectomy[15]. In the present study, FHRR and PHRR were used to determine the required procedure.

3D CT/^{99m}Tc-GSA SPECT fusion imaging

Three-phase dynamic CT was performed with a 320-row multidetector CT device (Aquilion ONE, Toshiba Medical Systems Co., Otawara, Japan). The obtained Digital Imaging and Communications in Medicine (DICOM) data were imported to the 3D image analysis system[16] (Volume Analyzer SYNAPSE VINCENT; Fuji Film Medical, Tokyo, Japan). 3D images were reconstructed from the DICOM data.

^{99m}Tc-GSA scintigraphy was performed separately from CT. Dynamic scanning was initially performed using a large-field view gamma camera (E.CAM; Siemens Japan, Tokyo) in an anterior view equipped with a low-energy high-resolution collimator, with the patient in the supine position after a bolus IV injection of 185 MBq of ^{99m}Tc-GSA. Dynamic planar images were obtained for 30 min by 147 serial frames (60 × 1 s, 87 × 20 s), with a matrix size of 128 × 128. Hepatic SPECT images were acquired after the dynamic study. The Digital Imaging and Communications in Medicine (DICOM) data obtained from SPECT were also imported to the SYNAPSE VINCENT and subsequently fused with the 3D CT images (Figure 1). PHRR and FHRR were calculated using the following formula:

Parenchymal hepatic resection rate (PHRR) (%) = liver resection volume/(total liver volume - tumor volume × 100)

Functional hepatic resection rate (FHRR) (%) = resection volume counts/total liver volume counts × 100

Statistical analysis

Statistical analyses were performed using JMP PRO version 11.2.0 (JMP statistical software; SAS Institute, Cary, NC, USA). The values are expressed as mean ± SDs. The correlation between PHRR and FHRR was assessed using Pearson's correlation product-moment coefficient. The ratio of FHRR to PHRR was calculated after stratifying the patients according to the presence or absence of preoperative therapies, macroscopic vascular invasion, and a tumor volume of 1000 mL. The ratio was derived by dividing the smaller value (PHRR or FHRR) with the larger value. The Wilcoxon signed rank test was used to compare data between groups.

RESULTS

Correlation between PHRR and FHRR

PHRR and FHRR were preoperatively calculated for all patients, and their correlation was determined (Figure 2). The mean PHRR and FHRR values were 44.5% ± 16.0% and 38.6% ± 19.9%, respectively. The FHRR value was approximately 10% smaller than the PHRR value, although there was a strong correlation between the two. The regression coefficient was 1.16 (P < 0.0001).

FHRR to PHRR ratio

There were 34 patients with preoperative therapies, including transcatheter arterial chemoembolization, radiation, radiofrequency ablation, chemotherapy, and partial resection), macroscopic vascular invasion, and/or a tumor volume of > 1000 mL, while 23 patients did not have any of these factors. The mean ratios for patients with and without these factors were 0.73 ± 0.19 and 0.82 ± 0.18, respectively (Figure 3). Thus, the ratio was significantly smaller for the former than for the latter (P < 0.05).

Surgical outcomes

Among the 57 patients, the most frequently performed procedure was right hepatectomy ± segment I (n = 18, 31.6%; Table 2). Biliary reconstruction was performed for 15 patients, including hepatopancreatoduodenectomy in two. Five patients exhibited Child-Pugh class B cirrhosis. These patients

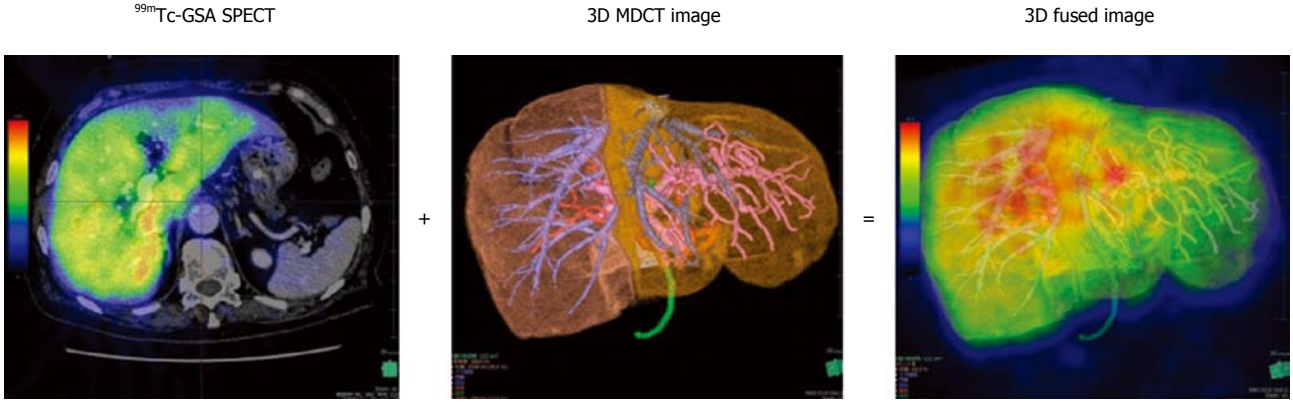

Figure 1 Creation of a 3D CT/^{99m}Tc-galactosyl human serum albumin single-photon emission computed tomography fusion image using the Volume Analyzer SYNAPSE VINCENT. The resection line is set using the 3D image reconstructed from Digital Imaging and Communications in Medicine (DICOM) data obtained from multidetector CT, following which the acquired ^{99m}Tc-GSA single-photon emission computed tomography (SPECT) image is fused with the 3D image. GSA: Galactosyl human serum albumin.

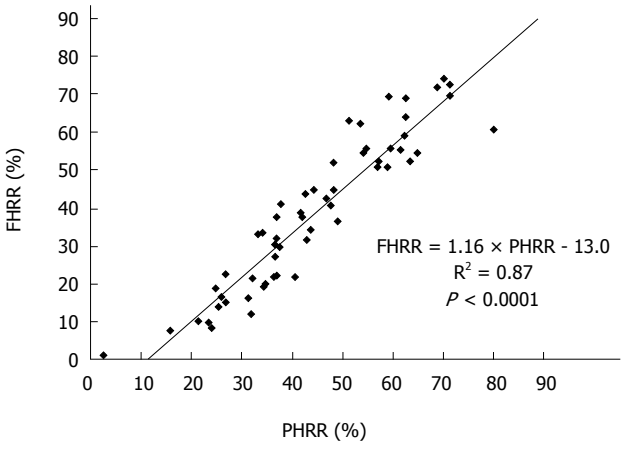

Figure 2 Correlation between the parenchymal hepatic resection rate and functional hepatic resection rate (calculated using 3D CT/^{99m}Tc-galactosyl human serum albumin single-photon emission computed tomography fusion imaging. Functional hepatic resection rate (FHRR) is strongly correlated with parenchymal hepatic resection rate (PHRR).

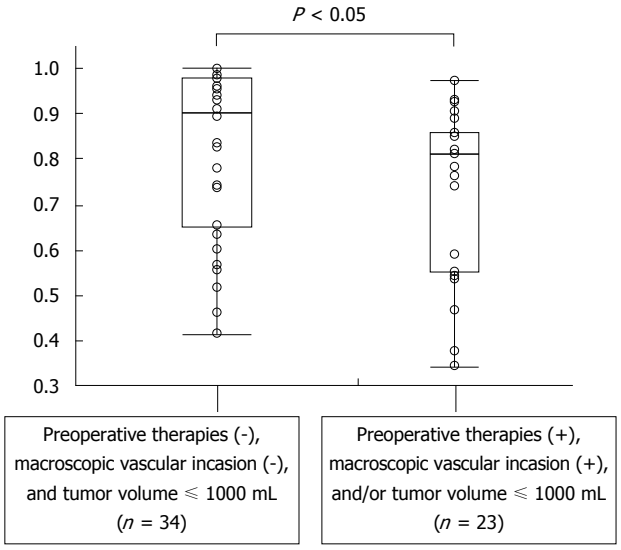

Figure 3 Comparison of the ratio of the functional hepatic resection rate to the parenchymal hepatic resection rate between patients with preoperative therapies, macroscopic vascular invasion, and/or a tumor volume of > 1000 mL and those without these factors. The ratio is significantly smaller for patients with preoperative therapies (transcatheter arterial chemoembolization, radiation, radiofrequency ablation, *etc.*), a tumor volume of > 1000 mL, and/or macroscopic vascular invasion than for those without these factors (0.82 ± 0.18 *vs* 0.73 ± 0.19, $P < 0.05$).

did not have chronic hepatitis, but they exhibited obstructive jaundice and malnutrition. Postoperative hyperbilirubinemia (serum total bilirubin ⩾ 5.0 mg/dL) was observed in six patients (10.5%). Five of these six patients underwent biliary reconstruction, and the remaining one exhibited preoperative cholangitis and underwent biliary drainage. PHLF, assessed according to the International Study Group of Liver Surgery definition, occurred in 12 patients (21.0%). Grade C liver failure was diagnosed in a patient who required repeat surgery because of portal vein thrombosis. Major morbidities (Clavien-Dindo grade ⩾ 3) occurred in 17 patients (29.8%). Bile leakage was a frequent occurrence (eight of 17 patients; 47.0%). There were no surgery-related deaths.

Representative case

A 34-year-old woman was diagnosed with a giant hemangioma (Figure 4). CT demonstrated that the posterior section was congested because of obstruction of the right hepatic vein by the giant hemangioma. Right trisectionectomy was planned. CT volumetry showed a tumor size of 2818 mL, a future remnant hepatic volume of 313 mL and a PHRR of 80.0%. 3D CT/^{99m}Tc-GSA SPECT fusion imaging showed an FHRR of 60.8%. She underwent right trisectionectomy without PVE and recovered without PHLF or any morbidity.

DISCUSSION

In the present study evaluating the significance of

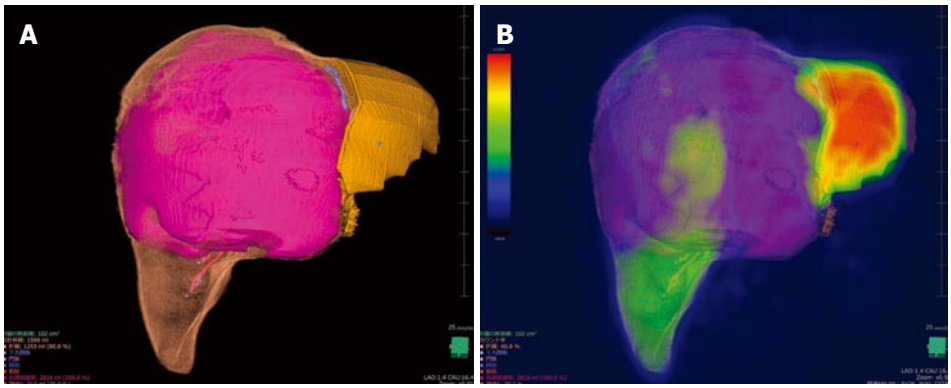

Figure 4 Representative case of a 34-year-old woman diagnosed with a giant hemangioma scheduled to undergo right trisectionectomy. A: CT volumetry showing the tumor volume as 2818 mL and parenchymal hepatic resection rate as 80.0%; B: 3D CT/^{99m}Tc-galactosyl human serum albumin single-photon emission computed tomography fusion image showing the functional hepatic resection rate as 60.8%. The patients underwent right trisectionectomy without preoperative portal vein embolization. She recovered without postoperative liver failure or any morbidity.

Table 2 Surgical outcomes *n* (%)

Hepatectomy procedure	
Right trisectionectomy	5 (8.8)
Extended right hepatectomy	2 (3.5)
Right hepatectomy ± segment Ⅰ	18 (31.6)
Central bisectionectomy	5 (8.8)
Left hepatectomy ± segment Ⅰ	16 (28.1)
Extended left hepatectomy ± segment Ⅰ	9 (15.8)
Left trisectionectomy ± segment Ⅰ	2 (3.5)
Biliary reconstruction	15 (26.3)
Surgical duration (min), mean ± SD	432 ± 167
Blood loss, median (g), range	430 (0-5700)
Red blood cell transfusion	18 (31.6)
Postoperative hyperbilirubinemia (T-bil ⩾ 5.0 mg/dL)	6 (10.5)
PHLF ISGLS grade (A/B/C)	9/2/1
Major morbidity (Clavien-Dindo grade ⩾ 3) (Ⅲa/Ⅲb)	12/5
Surgery-related death	0

T-bil: Serum total bilirubin, PHLF: Posthepatectomy liver failure, ISGLS: International Study Group of Liver Surgery.

FHRR calculated using 3D CT/99mTc-GSA SPECT fusion imaging, we found a strong correlation between FHRR and PHRR. However, the ratio of FHRR to PHRR was significantly smaller for patients with preoperative therapies, a tumor volume of > 1000 mL, and/or macroscopic vascular invasion than for those without these factors.

^{99m}Tc-GSA scintigraphy is a well-accepted modality for assessment of the hepatic functional reserve, along with the indocyanine green clearance test[7,8,17]. We previously reported the conversion formula for ^{99m}Tc-GSA scintigraphy data to the indocyanine green retention at 15 min[18]. This is useful for estimating the functional reserve of the whole liver, although the function of the future liver remnant can only be estimated from the volume calculated by CT volumetry. The introduction of 3D CT/^{99m}Tc-GSA SPECT fusion imaging has enabled precise estimation of the function of the focal liver lesion[11-14,19,20].

In the present study, PHRR and FHRR were calculated using 3D CT/^{99m}Tc-GSA SPECT fusion imaging along the accurate cutting line. FHRR was strongly correlated with PHRR, although they were discrepant for patients with preoperative therapies, macroscopic vascular invasion, and/or a tumor volume of >1000 mL. Theoretically, the discrepancy cannot occur without any deflection in liver function. Akaki *et al*[10] suspected that the decrease in the portal venous flow caused by the tumor is a major factor for the discrepancy between CT and ^{99m}Tc-GSA SPECT data. Mitsumori *et al*[21] reported that ^{99m}Tc-GSA SPECT findings were better correlated with the remnant liver function than CT findings because of the following reasons. First, the degree of hepatic dysfunction in the segment or lobe containing the hepatocellular carcinoma is greater than that in the segment or lobe without cancer. Second, the liver parenchyma surrounding the tumor is damaged by mechanical compression. Third, secondary liver damage may occur because of compression of the vessels and bile ducts by the tumor. We also hypothesized that preoperative therapies such as transcatheter arterial chemoembolization, radiofrequency ablation, and radiation induce focal liver damage, and that macroscopic vascular invasion and large tumors cause insufficient inflow or congestion. To prove the hypothesis, we compared the ratio of FHRR to PHRR between patients with and without the abovementioned factors and found that it was significantly smaller for patients with these factors. Thus, PHRR and FHRR are likely to be discrepant for patients with these conditions.

Recent advances in surgical techniques and pre- and postoperative care, including the decision criteria for hepatectomy and indications for liver resection, have been applied to extended hepatectomy. However, the famous decision criteria of Makuuchi[22] do not include the future remnant liver volume. A smaller remnant liver volume and an impaired hepatic functional capacity increase the risk of PHLF[4]; therefore, calculation of the future remnant hepatic

volume using CT volumetry is important[6]. It was reported that the type of surgery (more than or equal to hemihepatectomy *vs* less than hemihepatectomy) was associated with in-hospital mortality, and, specifically, patients who underwent less than a hemihepatectomy exhibited a mortality rate of 4.1%, while those who underwent more than or equal to hemihepatectomy exhibited a mortality rate of 6.5%[23]. Therefore, major hepatectomy requires detailed preoperative evaluations and excellent techniques. When FHRR is larger than PHRR, surgical decision making should be more meticulous to prevent PHLF and postoperative morbidities. It was reported that preoperative functional assessment by ^{99m}Tc-GSA SPECT is more useful than volumetric assessments by CT for the prediction of surgical outcomes[21,24]. On the other hand, when FHRR is smaller than PHRR, the indications for hepatectomy may be expanded beyond the safe limits. The reported safe limit of PHRR for a normal liver is 75%[25,26]. In the present study, a 34-year-old woman with a giant hemangioma uneventfully underwent right trisectionectomy. Although her PHRR was 80.0%, right trisectionectomy was accomplished without PVE after referring to an FHRR of 60.8%. This was probably because the function of the posterior section may have been deteriorated by congestion caused by obstruction of the right hepatic vein by the giant hemangioma. She recovered without PHLF or any morbidity. Further studies are required to confirm the safety of giving more importance to FHRR over PHRR.

In conclusion, we found an overall strong correlation between FHRR calculated using 3D CT/^{99m}Tc-GSA SPECT and PHRR in our study. However, FHRR and PHRR were discrepant for patients with preoperative therapies, macroscopic vascular invasion, and/or a tumor volume of > 1000 mL. These findings suggest that FHRR is useful for accurate estimation of the future liver remnant function and an important deciding factor for major hepatectomy.

COMMENTS

Background

Preoperative estimation of the hepatic functional reserve is important for major hepatectomy. Precise estimation of the future liver remnant function is difficult. ^{99m}Tc-galactosyl human serum albumin (^{99m}Tc-GSA) is frequently used for evaluating the hepatic functional reserve. ^{99m}Tc-GSA single-photon emission computed tomography (SPECT) can show the regional distribution of hepatic function. However, it is difficult to precisely estimate the functional reserve of the future liver remnant in patients requiring complicated resection. CT/^{99m}Tc-GSA SPECT fusion imaging enables the precise evaluation of hepatic function distribution, given the high spatial resolution provided by CT. In the present study, the authors calculated the functional hepatic resection rate (FHRR) using 3D CT/^{99m}Tc-GSA SPECT fusion imaging for patients undergoing major hepatectomy and determined its usefulness by determining its correlation with the parenchymal hepatic resection rate (PHRR).

Research frontiers

Several parameters using CT/^{99m}Tc-GSA SPECT fusion imaging, such as the preoperative total amount of receptor in the future remnant liver (R0-remnant), the liver uptake value corrected for body surface area [LUV(BSA)], and the uptake index (UI), have been reported to be useful for the preoperative estimation of remnant hepatic function, although they are considerably complex.

Innovations and breakthroughs

In the present study, the authors calculated the FHRR using 3D CT/^{99m}Tc-GSA SPECT fusion imaging and determined its usefulness. They considered the hepatic resection rate to be more intuitively useful compared with former reported parameters. A strong correlation between FHRR and PHRR in the present study was found. However, FHRR and PHRR were discrepant for patients with preoperative therapies, macroscopic vascular invasion, and/or a tumor volume of > 1000 mL.

Applications

This study suggests that the measurement of FHRR is particularly useful for patients with preoperative therapies, macroscopic vascular invasion, and/or a tumor volume of > 1000 mL.

Terminology

^{99m}Tc-GSA scintigraphy: An image inspection used for evaluating the hepatic functional reserve. ^{99m}Tc-GSA SPECT: A procedure to obtain tomographic images of ^{99m}Tc-GSA scintigraphy.

Peer-review

The manuscript is well organized and developed. The study is inserted in the wide topic of selection of the patients for hepatic surgery, first of all based on the evaluation of the functions of the remnant liver.

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ORIGINAL ARTICLE

Retrospective Study

Clinicopathological features of familial adenomatous polyposis in Korean patients

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Abstract

AIM: To identify prognostic factors and to correlate *APC* mutations with clinical features, including extracolic manifestations.

METHODS: One hundred thirty-five patients who underwent surgical procedures for familial adenomatous polyposis (FAP) were included. FAP was diagnosed when the number of adenomatous polyps was > 100. Data related to patient, extracoloic manifestations, cancer characteristics, operative procedure, follow up and surveillance were collected. *APC* mutation testing was performed in the 30 most recent patients. DNA was extracted from peripheral blood and polymerase chain reaction products using 31 primer pairs on *APC* gene were sequenced. A retrospective study was performed to investigate a causal relationship between prognosis and feature of patient.

RESULTS: The mean age of the 51 patients with colorectal cancer (CRC) was older than that of those without CRC (30.5 *vs* 36.9, P = 0.002). Older individuals were more likely to have colon cancer at the time of FAP diagnosis [odds ratio, 4.75 (95%CI: 1.71-13.89) and 5.91(1.76-22.12) for 40-49 years and age > 50 *vs* age < 30). The number of confirmed deaths was 13 and the median age at death was

40 years (range, 27 to 85 years). Ten of the deaths (76.9%) were from CRC. Another cause of two cases of death were desmoid tumors (15.4%). Development of cancer on remnant rectal or ileal mucosa after surgery was not observed. The *APC* mutation testing revealed 23 pathogenic mutations and one likely pathogenic mutation, among which were four novel mutations. The correlation between mutational status and clinical manifestations was investigated. Mutations that could prodict poor prognosis were at codon 1309 which located on mutation cluster region, codon 1465 and codon 1507.

CONCLUSION: Identification of *APC* mutations should aid in the diagnosis and counseling of family members in terms of early diagnosis and management of FAP.

Key words: Adenomatous polyposis coli; Colorectal neoplasms; *APC* gene; Prognosis; Survival

Core tip: Diagnostic delay to make adequate management harder, resulting in advanced colorectal cancer and accompanying morbidities, was not uncommon in patients with familial adenomatous polyposis. This study investigated prognostic factors and the correlation between *APC* mutations and clinical features, including extracolic manifestations. The present study revealed that early diagnosis and management of high-risk patients is essential and suggests the necessary *APC* mutations testing in the diagnosis and counseling of patients by informing on disease prognosis.

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INTRODUCTION

Familial adenomatous polyposis (FAP) is an autosomal dominantly inherited cancer syndrome caused by germ-line mutation of the adenomatous polyposis coli gene (*APC*) and characterized by the development of 100 to 1000 colorectal adenomatous polyps in its classic form[1]. Subjects with FAP have a risk of almost 100% of developing colorectal cancer by 40-50 years of age[2]. The extracolic phenotype is characterized by the development of adenomatous polyps in the upper gastrointerstinal tract, gastric fundic polyps, desmoid tumors, osteomas, fibrous bone dysplasia, skin fibromas, pineal blastomas, brain cysts, papillary thyroid cancers (PTCs) and congenital retinal pigment epithelial hypertrophy (CHRPE). Colorectal cancer is the most common cause of death in FAP patients and desmoid tumors are the second[3]. To prevent the development of colon cancer, prophylactic removal of the entire colorectal mucosa is recommended for FAP patients.

The FAP registry was performed to improve the prognosis of FAP through early diagnosis and surgery before the development of colorectal cancer[4]. In addition, a recent molecular genetic test for *APC* mutations makes it possible to detect asymptomatic or not developing polyposis patients. *APC* mutations can be detected in 80%-90% of patients who have a valid indication for the test[5]. Since most families are managed through hospital-based registries and not by the nation wide registry, there are few investigations of integrative and continuous follow-up to provide treatment guidelines for Korean families. Patients' indifference and diagnostic delay appear to make adequate management harder, resulting in advanced colorectal cancers and accompanying morbidities.

The purpose of this study was to describe the clinicopathological features of the disease and the expression of extracolic manifestations, and to evaluate whether *APC* mutations have prognostic value in Korean patients. Finally, we propose management options for high-risk patients to improve outcome.

MATERIALS AND METHODS

Patient enrollment and eligibility

FAP was defined by the presence of more than 100 colorectal adenomatous polyps, according to intraoperative findings and pathologic reports. In this retrospective study, 135 consecutive patients who underwent surgical procedures for FAP at the Asan Medical Center (Seoul, Korea) between August 1991 and July 2014 were included with median follow-up periods of 54 mo. Patients with fewer than 100 adenomatous polyps and patients with hamartomatous polyposis were excluded. Demographics and length of follow-up were documented for each patient.

Work-up to surveillance

The extent of disease and developing cancer was assessed by colonoscopy, abdominopelvic computed tomography (CT) or magnetic resonance imaging (MRI). Positron emission tomography-computed tomography (PET-CT) was performed if advanced colorectal cancer was suspected. Investigation of extra-colonic manifestations was done when FAP was diagnosed or in the immediate postoperative period. Tests included physical examination for skin lesions and palpable masses, X-ray imaging for bone lesions, dental and ophthalmic examination and gastroduodenal endoscopy.

Patients were subjected to one of the following procedures: total proctocolectomy with ileal pouch anal anastomosis (TPC/IPAA) with hand sewn or

Table 1 Main characteristics of the patients with or without colorectal cancer (*n* = 135)

	Total (*n* = 135)	with CRC (*n* = 51)	without CRC (*n* = 84)	*P* value[1]
Age distribution[2]	31 (15-78)	34 (15-78)	28 (15-64)	0.002
< 20	11 (8.2)	5 (9.8)	6 (7.1)	
20-39	86 (63.7)	23 (45.1)	63 (75.0)	
40-59	34 (25.2)	21 (41.2)	13 (15.5)	
⩾ 60	4 (3.0)	2 (4.0)	2 (2.4)	
Sex ratio (M:F)	1:0.78	1:0.59	1:0.91	0.239
CRC FHx	72 (53.3)	20 (39.2)	52 (61.9)	0.010
FAP FHx	39 (28.9)	7 (13.7)	32 (38.1)	0.003
Malignancy FHx	86 (63.7)	28 (54.9)	59 (70.2)	0.071
Follow-up (mo)[2]	54.2 (1-271)	55.5 (1-271)	53.49 (1-212)	0.855
Alive at follow-up	122 (90.4)	40 (78.4)	82 (97.6)	< 0.001
No. of polyps[2]	435	427	440	0.866
OP procedures				0.846
Handsewn TPC/IPAA	84 (62.2)	29 (56.8)	55 (65.5)	
Stapled TPC/IPAA	33 (24.4)	10 (19.6)	23 (27.4)	
TPC/end ileostomy	8 (5.9)	8 (15.7)	0	
TC	10 (7.4)	4 (7.8)	6 (7.1)	
Mortality	13 (9.6)	11 (21.6)	2 (2.9)	< 0.001
Cancer specific	10 (7.4)	10 (19.6)	0	
Other reason	3 (2.2)	1 (2.0)	2 (2.4)	

[1]Pearson χ^2 test or Fisher's exact test for categorical variable, *t*-test for continuous variable; [2]Data are expressed as median (range). Data are expressed as number (%) unless otherwise stated. CRC: Colorectal cancer; FAP: Familial adenomatous polyposis; FHx: Family history; TPC/IPAA: Total proctocolectomy with ileal pouch anal anastomosis; TC: Total colectomy.

stapled anastomosis, TPC with end ileostomy, and total colectomy with ileorectal anastomosis (TC/IRA). The patients who underwent TPC/IPAA with mucosectomy with hand sewn anastomosis or TPC with end ileostomy did not have any remnant rectal mucosa. By contrast, the TPC/IPAA with stapled anastomosis or TC/IRA procedures left remnant rectal mucosa.

Annual colonic and gastric endoscopic surveillance is recommended after surgery. Colonoscopy was performed using a standard adult colonoscope or a gastroscope. Any lesion suspected to be an adenoma during endoscopic surveillance was biopsied. If the polyps could not be controlled by endoscopic procedures, surgical procedures were performed. Surveillance endoscopies and associated pathologic reports were reviewed including the incidence, timing and histology of the adenoma and cancer development. In patients with colon cancer, a follow-up assessment was performed every 3 to 6 mo for the first 2 years and annually thereafter.

APC mutation

In our institution, *APC* germ-line mutation testing has been performed since 2008 in the clinical setting to diagnosis FAP and counsel patients and their family members. The 30 most recent patients among 135 FAP patients underwent mutation tests. Genomic DNA was extracted from peripheral blood using a Qiagen DNA extraction kit (Qiagen, Hilden, Germany). Thirty-one primer pairs were used, including primer pairs covering exons 1-14 and 17 primer pairs covering exon 15 of the *APC* gene. Polymerase chain reaction (PCR) was performed using Accu-Power PCR premix (Bioneer, Daejeon, Korea). Each PCR product was sequenced using a 3130xl or 3730 genetic analyzer (Applied Biosystems, Foster City, CA). Sequences were aligned using the Sequencher 4.9 software (Gene Codes Corporation, Ann Arbor, MI).

Statistical analysis

Continuous variables were described as mean ± SD or median with ranges and categorical variables were expressed as numbers and percentages. Differences in characteristics of patients with or without colorectal cancer were calculated using *t*-tests for continuous variables and χ^2 tests or Fisher's exact tests for categorical variables. Survival curves were generated by the Kaplan-Meier method, and univariate survival distributions were compared with the use of the log-rank test.

All statistical analyses were carried out using SPSS version 20.0 (IBM Corp., Armonk, NY, United States) and R version 3.1.2 (The Comprehensive R Archive Network: http://cran.r-project.org). The level of significance was set at $P < 0.05$.

RESULTS

Characteristics of patients

The characteristics of the 135 patients are shown in Table 1. There was no significant difference in age at diagnosis between men and women ($P = 0.613$). The mean age of patients with cancer was significantly greater than that of patients without cancer ($P = 0.002$) and the mean age of the patients with cancer at an early stage (carcinoma *in situ* and carcinoma restricted to the submucosal layer), 32.7 years, was between the mean age of the patients with advanced cancer (38.7 years) and that of the patients without cancer. Thirty six of the 39 patients who had a family history of FAP also had a family history of colorectal cancer except three patients who only have a family history of FAP. The rates of familial history of FAP or CRC were significantly higher in patients without CRC than in patients with CRC. Only 12 of the 135 patients had been diagnosed with FAP prior to surgery and the median follow-up period was 5 years. TPC with end ileostomy was performed in patients with rectal cancer in 8 cases and total colectomy was performed in patients who presented a few rectal polyps (less than 5 and endoscopically removable) in 10 cases. There was no significant difference in operative procedure between patients with CRC and patients without CRC. Eighty-eight (65.2%) patients underwent a colonoscopy because of gastrointestinal

Table 2 Initial symptoms and extracolic manifestations (*n* = 135)

		n (%)
Chief complaint	Hemotochezia	34 (25.2)
	Diarrhea	23 (17.0)
	Abdominal pain	14 (10.4)
	Constipation	8 (5.9)
	Abdominal mass	2 (1.5)
	None	47 (34.8)
	Others	7 (5.2)
Extracolic manifestation	Fundic polyps	57 (42.2)
	UGI adenoma	46 (34.1)
	CHRPE	29/100 (29.0)
	Desmoid tumor	20 (14.8)
	Dental anomaly	11/96 (11.5)
	Osteoma	9/84 (10.7)
	Epidermoid cyst	7 (5.2)
	Papillary thyroid cancer	6 (4.4)
	Ampulla of vater adenoma	5 (3.7)
	Adrenal adenoma	4 (3.0)
	Gastric cancer	4 (3.0)

UGI: Upper gastrointestinal; CHRPE: Congenital retinal pigment epithelial hypertrophy.

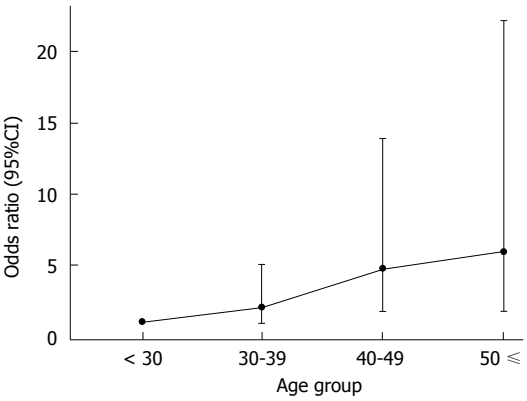

Figure 1 Odds ratio for having colon cancer at the time of familial adenomatous polyposis diagnosis beween age group, compared to age < 30 yr.

symptoms at the time of diagnosis and 47 patients (34.8%) underwent a colonoscopy because of positive stool occult blood test or health medical examination without specific symptom (Table 2).

Extra-colonic features in FAP

Extra-colonic manifestations are described in Table 2. Gastric polyps were found in 63 (46.7%) patients. The histological types of gastric polyps were fundic gland polyp in 57 patients (90.5%) and tubular adenoma biopsied from the antrum of the stomach in 16 patients (25.4%). Ten patients had both fundic gland polyps and tubular adenomas. Four cases of early gastric cancer were diagnosed and the median age was 41 (range, 34-51 years). Two of them were treated by endoscopic submucosal excision and two underwent distal gastrectomy. Among six patients with papillary thyroid cancer (PTC), two were diagnosed with PTC and underwent total thyroidectomy before receiving a diagnosis of FAP. Five patients (3.7%) underwent ampullectomy for polyps in the ampulla of vater in follow-up periods, and high-grade dysplasia was detected in one patient. Desmoid tumors were diagnosed at surgery in four patients and developed in 16 other cases during follow-up; the median age at diagnosis of desmoid tumors was 31 (range, 20-47 years). Desmoid tumors were mostly intra-abdominal in 19 cases, particularly mesenteric with one in the abdominal wall. The postoperative risk for desmoid tumors was not different between men and women (P = 0.625).

Table 3 Clinicopathologic characteristics of the patients with colorectal cancer (*n* = 51)

	n (%)
Tumor location	
Colon	32 (62.7)
Rectum	19 (37.3)
Synchronous lesion	24 (47.0)
Definitive stoma	8 (15.7)
Neoadjuvant treatment	2 (3.9)
Tumor grade	
WD-MD	46 (88.2)
PD	5 (11.8)
Final pathological staging	
Tumor size (mm)[1]	41 (2.44)
LVI	12 (23.5)
PNI	8 (15.9)
pT0-1	15 (29.4)
pT2	6 (11.8)
pT3	25 (49.0)
pT4	5 (9.8)
Lymph node invasion	25 (49.0)
Distance metastasis	7 (13.7)
Staging	
0-1	18 (35.3)
2	6 (11.8)
3	20 (39.2)
4	7 (13.7)
Local relapse	0
Systemic relapse	13 (25.5)

[1]Data are expressed as mean (standard deviation). CRC: Colorectal cancer; WD: Well differentiated; MD: Moderately differentiated; PD: Poorly differentiated; pT: Pathologic T status; LVI: Lymphovascular invasion; PNI: Perineural invasion.

Colonic features and development of cancer

Clinicopathologic characteristics are shown in Table 3. The incidence of synchronous lesions and the rates of early colorectal cancer were relatively high. Odds ratio for having colon cancer at the time of FAP diagnosis for 40s and over 50s years were 4.75 (95%CI: 1.71-13.89) and 5.91 (95%CI: 1.76-22.12) when compared with under the age of 30 (Figure 1). Five cases of colorectal cancer were diagnosed in patients aged less than 20 years. Four of them had carcinoma *in situ* and one showed invasion to proper muscle of rectal wall without lymph node metastasis. Among 60 of *de novo* patients , who did not have a family history of colorectal cancer or FAP, 31 presented colorectal cancer

Table 4 Genotype-phenotypic correlations for unrelated Korean patients

Exons (introns, I)	Nucleotide change	AA change	Mutation type	Age of diagnosis	CRC	Patient characteristics			
						No. of polyps	CHRPE	PTC	DT
7	778C>T	p.Gln260X	Nonsense	49	No	400			
10	1378G>T[1]	p.Glu460X	Nonsense	21	No	1300	O		
10	1381G>T[1]	p.Glu461X	Nonsense	23	No	400			O
11	1495C>T	p.Arg499X	Nonsense	28	Yes	500			
13	1690C>T	p.Arg564X	Nonsense	54	Yes	400			
15	2797-2800delAACA	p.Asn933LeufsX21	Frameshift	29	No	300	O		
15	2805C>G	p.Tyr935X	Nonsense	32	Yes	600	O	O	
15	3183-3187delACAAA	p.Lys1061fsX	Frameshift	28	No	400	O		
15	3183-3187delACAAA	p.Lys1061fsX	Frameshift	12	No	800			
15	3505-3509delGAGAA	p.Glu1169ThrfsX8	Frameshift	38	No	700	O		
15	3523C>T	p.Gln1175X	Nonsense	22	No	1400	O		
				20	No	450	O		
15	3578delA	p.Gln1193ArgfsX72	Frameshift	34	No	100			
15	3595-3596delAA	p.Lys1199GlufsX8	Frameshift	28	No	100	O	O	
15	3631-3632delAT	p.Met1211fsX5	Frameshift	31	No	200			
15	3709C>T	p.Gln1237X	Nonsense	20	No	100	O		
15	3925-3928delGAAA[2]	p.Glu1309ArgfsX11	Frameshift	30	Yes	200	O	O	O
15	3927-3931delAAAGA[2]	p.Glu1309AspfsX4	Frameshift	17	Yes	2500			
15	4393-4394delAG	p.Ser1465TrpfsX3	Frameshift	31	No	130			O
15	4393-4394delAG	p.Ser1465TrpfsX3	Frameshift	31	No	360			O
15	4519-4520insTGAGCTCA[1]	p.Ser1507MetfsX6	Frameshift	26	Yes	1000	O		
15	4782-4785delAGCC	p.Ala1595ArgfsX54	Frameshift	41	Yes	100			
(I 4)	532-2A>C		Splice-site	39	Yes	100			
(I 4)	532-2A>C		Splice-site	38	No	100			
(I 14)	1958+3A>T[1]		Splice-site	19	No	740			

[1]*APC* mutation identified as novel mutations; [2]*APC* mutation located in MCR. CHRPE: Congenital retinal pigment epithelial hypertrophy; CRC: Colorectal cancer; PTC: Papillary thyroid cancer; DT: Desmoid tumor.

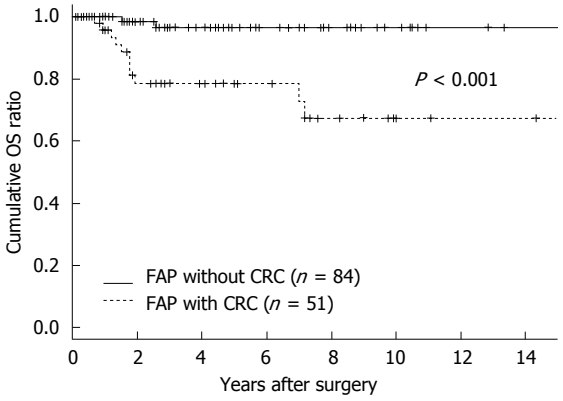

Figure 2 Overall survival of patients with familial adenomatous polyposis grouped by accompanying based on presence of colorectal cancer to none development of colorectal cancer. CRC: Colorectal cancer.

and it is significantly higher than that of patients with family history of FAP or CRC (51.7% *vs* 27.8%, P = 0.013), but there was no significant difference in mean age of development of CRC between the two groups (P = 0.265). Patients with gastrointestinal symptoms were more frequently diagnosed with cancer than patients without symptoms and the difference was statistically significant (P = 0.042). Cancer patients without symptoms at the time of diagnosis had a higher incidence of early stage cancer than those with symptoms at diagnosis (P = 0.041).

The patients who have remnant rectum after rectal excision (68.1%) presented a significantly higher incidence of adenomatous polyps in remnant rectal mucosa than other patients underwent TPC and mucosectomy (P < 0.001). Development of cancer on remnant rectal mucosa was not observed, but one patient who underwent TPC/IPAA with stapled anastomosis was diagnosed with villotubular adenoma with high-grade dysplasia and underwent trans-anal excision. There was no significant difference in the incidence of adenomatous polyps in pouch or ileum regardless of operative procedure (P = 0.465) and malignant change or dysplasia was not observed on any ileal adenomas.

Confirmed deaths were observed in 13 patients with a median age at death of 40 years (range, 27-85 years). Deaths from colorectal cancer accounted for 10 cases (76.9%). In these 10 patients, five cases had distant metastasis at the time of diagnosis. Comparison of postoperative survival between patients with colorectal cancer and patients without cancer is shown in Figure 2. In patients without CRC, only two deaths (15.4%) were observed in patients with desmoids tumor causing major complication and there was no death caused by desmoid tumors in the CRC group.

APC mutation test

The germ-line *APC* mutations that were detected in these patients are listed in Table 4 with clinical details.

Two of them was a sister relationship, so 29 unrelated patients were included. Testing revealed 23 pathogenic mutations and one likely pathogenic mutation. Five patients were negative for *APC* mutations. Of the 24 pathogenic or likely pathogenic mutations, frameshift, nonsense and splice-site mutations represented 54.2%, 33.3% and 12.5%, respectively. Sixteen of 24 mutations were localized within exon 15. Just one mutation in codon 1309 was located within the mutation cluster region (MCR, mutations from codon 1250 to 1464). The mutations on codons 1061, 1309 and 1465 were frequently observed in two cases, respectively. Four novel mutations were identified based on the Human Gene Mutation Database (www.hgmd.cf.ac.uk/ac): c.1378G>T, c.3709C>T, c.4519-4520insTGAGCTCA and c.1958+3A>T. A patient with a mutation on codon 461 (c.1378G>T) presented with a desmoid tumor and a upper gastrointerstinal (UGI) adenoma. A man with a novel mutation of codon 1507 (c.4519-4520insTGAGCTCA) was diagnosed with advanced synchronous colorectal cancer and had a family history of colorectal cancer; his father died of colon cancer with liver metastasis at 35 years of age. His older brother also died of colon cancer which was not indicated for surgery at 19 years of age. His younger brother was diagnosed with colon cancer and underwent surgery at age 19, but died of multiple liver metastases at 20 years of age. He presented with CHRPE, dental anomaly and adrenal adenoma.

DISCUSSION

While CRC is the inescapable fate of untreated FAP patients, few studies provide information on the age distribution at the time of diagnosis of CRC in FAP since most cases are diagnosed in a premalignant stage. In this study, CRC occurred in more than one-third of FAP patients. The prevalence of CRC in *de novo* patients was significantly higher than that of patients with a family history of FAP or CRC, but there was no significant difference in the mean age of development of CRC between the two groups. The mean age at diagnosis of FAP was significantly different between patients with CRC and those without CRC. In the present study, the prevalence of CRC in patients with a family history was more frequent than that of a previous study for call up patients who diagnosed FAP through family screening[6] since most patients underwent a colonoscopy after developing intestinal symptoms despite having a family history of CRC or FAP. This means that development of CRC in FAP patients depends more on the age at diagnosis than on family history and that we have the opportunity to reduce the frequency of CRC, especially in patients with a family history. We found that diagnosis of colorectal cancer exceeded 10% at the age of 26 and 50% at the age of 43 and that this age distribution was similar to that described in a previous Western report[7]. We also found that there were no cancer-specific deaths in early cancer cases (carcinoma *in situ* and cancer restricted to the submucosa). Even though some cases of CRC before 20 years of age were observed in this study, all were diagnosed earlier than stage Ⅰ and survived without recurrence. To improve survival, a reduction in the incidence of CRC at diagnosis should be achieved. These results suggest that a diagnosis of FAP should be obtained before the early twenties.

The standard prophylactic procedure for the majority of patients with FAP is TPC with IPAA. In selected patients, rectum-preserving surgery might be indicated. We performed TC with IRA in six patients who presented with a few rectal polyps. All of them developed adenomas on remnant rectal mucosa but did not develop any carcinomas. The cumulative risk of rectal cancer after IRA was reported to be about one-fourth of patients after 15-25 years despite surveillance[8]. Therefore continuing endoscopic surveillance is necessary for these patients. The incidence of developing adenomatous polyps after TPC in the anorectal transient zone was reported as 10%-15%[9]. There are several reports of adenomatous polyps and cancers arising from the ileal pouch mucosa as opposed to the anastomotic site[10]. We found nine cases of adenomatous polyps arising from the ileal pouch mucosa but did not observe cancer or dysplasia. Development of cancer on remnant rectal mucosa was also not observed, but one patient among 18 cases of adenomatous polyps arising from remnant mucosa was diagnosed with villotubular adenoma with high-grade dysplasia and underwent trans-anal excision. The incidence of tubular adenoma in remnant mucosa and ileal pouch increased over time, so periodical follow-up was necessary.

In the present study, gastric polyps were a common manifestation of FAP. The histological types of gastric polyps were mostly fundic gland polyps and secondary tubular adenomas biopsied from the antrum of the stomach. This is consistent with previous reports documenting that fundic gland polyps are the most common gastric polyps and that adenoma is the second most prevalent gastric lesion in individuals with FAP[11]. We observed that three patients had asymptomatic gastric carcinoma at their first gastroduodenal endoscopy while one developed carcinoma during the surveillance study 18 years after surgery and one developed TA with high-grade dysplasia (HGD) 10 years after surgery, which was treated with endoscopic submucosal dissection. All of these cancers and HGD developed in the antrum. The overall incidence of 3% for gastric carcinoma in this study is similar to the high prevalence of gastric cancer reported in previous study[12]. Nonetheless, the prevalence of gastric cancer in FAP was not reported to be higher than that in the general population in Western countries and the most common gastric cancer site in Western reports was the fundus of the stomach (fundic gland polyps)[11,13]. The high incidence

of gastric cancer and the different sites may indicate inter-ethnic differences in the presentation of FAP. In contrast to Western reports of a relatively high incidence of duodenal cancer, we did not observe any malignant neoplasms on the duodenum[14]; however, it is important to consider the possibility of malignancy on the duodenum, since duodenal adenomatous polyps occurred in one-third of patients and since one case of HGD was detected on the ampulla of vater in our study.

We found that desmoid tumor was the only cause of death associated with FAP except caused by CRC in this study. The incidence of 14.8% and location of intra-abdominal site, particularly small bowel mesentery, in the present study is consistent with previous studies[15]. In particular, mutation on codons 1445-1580 is associated with postoperative development of desmoid tumors and recommended to postpone elective colectomy[16], but this correlation does not always appear to be consistent. In our study, four of 20 desmoid tumors developed without any history of surgical trauma. Desmoid tumors unrelated to surgical trauma are a relatively poor prognostic factor[17]. In two of four synchronous desmoid tumors, the same *APC* mutation (codon 1465; c.4393-4394delAG), reported as a desmoid tumor-associated mutation[18], was detected in unrelated patients. The mutation at codon 1465 located in exon 15 may implicate progression, so patients having this mutation and desmoid tumors should be considered for permanent stoma at the time of primary surgery. Other mutations associated with desmoid tumors in our study were at codons 461 (c.1381G>T) and 1309 (c.3925-3928delGAAA). The incidence of desmoid tumors increased over time, so periodic follow-up is necessary.

Another feature of FAP is the variation in clinical course between patients, so prediction of the severity of the disease is important in the interest of effective cancer prevention. Correlation between mutation and the age at onset of intestinal symptoms and development of CRC was reported[19], but the manifestation may be variable even in patients with identical germ-line mutations. Tumors with mutations localized in exon 15 between codons 1250 and 1464 (mutation cluster region or MCR) have generally a worse prognosis with early onset of the disease and may be candidates for IPAA due to the high incidence of secondary protectomy after primary colectomy[19,20]. In the present study, two kinds of mutations at codon 1309 within the MCR were detected: c.3925-3928delGAAA and c.3927-3931delAAAGA. Deletion of 5 base pairs at codon 1309 (c.3927-3931delAAAGA), the most common mutation, was detected in a patient who presented with CRC at age 17. This finding corresponds with previous results showing that mutation at codon 1309 (c.3927-3931delAAAGA) is associated with early development of intestinal symptoms and death from colorectal cancer[19]. The incidence of this mutation was 4% in our study and 29% in a previous study[16] and its frequency varied. No other MCR mutation was observed in the present study (Table 4). These findings implicate the possibility of ethnic differences in *APC* mutations. We also observed one interesting novel mutation at codon 1507 within exon 15, c.4519-4520insTGAGCTCA, which was not located in the MCR but presented in a patient with early onset and poor prognosis of CRC. This information may help support surgical decisions of method and timing. In the present study, two-thirds of *APC* mutations were localized to exon 15. Two of the most common mutations were also identified in this study: at codon 1061 (c.3183-3187delACAAA) and codon 1465 (c.4393-4394delAG). Mutation at codon 1061 has not been reported in Korean patients even though it is one of the most frequently reported mutations.

Extracolic manifestations showed correlations with *APC* mutations. The expression of CHRPE is associated with a clearly distinct region of mutations located between codons 311 and 1445 of *APC*[20,21]. All mutations detected with CHPRE in this study were located on reported distinct region but one mutation was not located within this region (codon1507; c.4519-4520insTGAGCTCA). It is necessary to observe carefully the clinical manifestation of this novel mutation. PTC affects 1%-2% of patients with FAP. PTC is associated with a mutation located between codons 140 and 1309 of *APC*, and a strong association with CHRPE also exists[21]. In the present study, three mutations on codons 935, 1199 and 1309 were detected and all of them presented CHRPE, which is consistent with previous reports. The mutation detection rate in our study is consistent with previous reports[5]. None of the cases can be explained by large allelic deletions, promoter deletions, deep intronic base changes and chromothripsis in germline[22], reduced or absent expression from one allele of *APC* or bi-allelic germ-line mutation of *MYH*[23].

A major limitation of this study was the relatively small number of patients and the fact that the study was based on one medical center, so the result may not be generalizable. A multicenter or nationwide registry investigation is needed. Secondly, the retrospective nature of the study and use of medical records for data collection prevented knowledge about affected family members because of unclear medical records and low rates of hospital utilization in the previous two generations in Korea. A prospective cohort study using the FAP registry is required. Finally, the *APC* mutation test has been used only recently in suspected patients in the clinical setting since gene analysis only became available in 2008 at our institute. Mutation testing should be conducted in patients who do not know their family history of FAP to identify affected members.

In conclusion, we reported clinicopathologic features and prognosis of FAP in Korean patients. The

data suggest the necessity for early diagnosis and management of high-risk patients. *APC* mutations can inform on the severity of the disease and on prognosis and identify family members having the same mutation. The identification of *APC* gene mutations and the understanding of genotype-phenotype correlations should aid in the diagnosis and counseling of patients.

COMMENTS

Background

Familial adenomatous polyposis (FAP) have a risk of almost 100% of developing colorectal cancer by 40-50 years of age. Colorectal cancer is the most common cause of death in FAP patients and desmoid tumors are the second. A recent molecular genetic test for *APC* mutations makes it possible to detect asymptomatic or not developing polyposis patients. Studies on clinicopathological features of the disease and clinical availability of *APC* mutations testing were performed in order to improve the early detection and management. Diagnostic delay makes adequate management harder in patients with FAP. This study investigated prognostic factors and the correlation between APC mutations and clinical features.

Research frontiers

Diagnostic delay makes adequate management harder in patients with FAP. This study investigated prognostic factors and the correlation between *APC* mutations and clinical features.

Innovations and breakthroughs

The present study revealed that early diagnosis and management of high-risk patients is essential and suggests the necessary APC mutations testing in the diagnosis and counseling of patients by informing on disease prognosis.

Applications

The type of APC mutation was one indicator that could be used to evaluate the poor prognosis of patients with FAP. This finding suggested that APC mutation testing can inform on the severity of the disease and on prognosis and identify family members having the same mutation.

Terminology

APC gene located on chromosome 5q21-22 and found to be mutated in FAP patients. This gene encodes a tumor suppressor protein (APC protein, 2843 amino acids). APC protein acts as an antagonist of the Wnt signaling pathway and also play a role in process of cell migration and adhesion, transcriptional activation, and apoptosis.

Peer-review

It's a very nice and significant work. Congrats and thanks to authors for meaningful success. Congrats for authors for writing so much nice topic.

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ORIGINAL ARTICLE

Clinical Trials Study

Efficacy and safety of granulocyte, monocyte/macrophage adsorptive in pediatric ulcerative colitis

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Informed consent statement: This investigation was conducted in accordance with GCP, the Declaration of Helsinki and EN ISO 14155:2003. All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

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Abstract

AIM: To investigate efficacy and safety for granulocyte, monocyte apheresis in a population of pediatric patients with ulcerative colitis.

METHODS: The ADAPT study was a prospective, open-label, multicenter study in pediatric patients with moderate, active ulcerative colitis with pediatric ulcerative colitis activity index (PUCAI) of 35-64. Patients received one weekly apheresis with Adacolumn® granulocyte, monocyte/macrophage adsorptive (GMA) apheresis over 5 consecutive weeks, optionally followed by up to 3 additional apheresis treatments over 3 consecutive

weeks. The primary endpoint was the change in mean PUCAI between baseline and week 12; the secondary endpoint was improvement in PUCAI categorized as (Significant Improvement, PUCAI decrease of $\geqslant$ 35), Moderate Improvement (PUCAI decrease of 20 < 35), Small Improvement (PUCAI decrease of 10 < 20) or No change (PUCAI decrease of < 10).

RESULTS: Twenty-five patients (mean age 13.5 years; mean weight 47.7 kg) were enrolled. In the intention-to-treat set (ITT), the mean value for PUCAI improvement was 22.3 [95%CI: 12.9-31.6; *n* = 21]. In the per-protocol (PP) set, the mean improvement was 36.3 [95%CI: 31.4-41.1; *n* = 8]. Significant Improvement was recorded for 9 out of 20 patients (45%); 5 out of 20 patients (25%) had Moderate Improvement and one patient (5%) had No Change in PUCAI score at week 12. In the PP set, six out of eight patients (75%) showed Significant Improvement; and in two out of eight patients (25%) Moderate Improvement was recorded. The endoscopic activity index (EAI) decreased by 3 points on average. Seven (7) out of 21 (33%) patients in ITT and 4 out of 8 (50%) patients in PP have used steroids during the clinical investigation. The mean steroid dosage for these patients in the ITT set decreased from a mean 12.4 mg to 10 mg daily on average from Baseline to week 12.

CONCLUSION: Adacolumn® GMA apheresis treatment was effective in pediatric patients with moderate active Ulcerative Colitis. No new safety signals were reported. The present data contribute to considering GMA apheresis as a therapeutic option in pediatric patients having failed first line therapy.

Key words: Granulocyte-monocyte apheresis; Pediatric; Ulcerative colitis; Inflammatory bowel disease; Therapy; Steroids; Clinical trial

Core tip: For a considerable group of children with ulcerative colitis (UC), treatment options are limited especially after failure of conventional treatment. The ADAPT trial was designed to generate prospective cohort data on efficacy and safety levels in moderate active pediatric UC patients when treated with Adacolumn granulocyte, monocyte/macrophage adsorptive (GMA). The present data contribute to considering GMA apheresis as a therapeutic option in pediatric patients having failed first line therapy.

Ruuska T, Küster P, Grahnquist L, Lindgren F, Wewer AV. Efficacy and safety of granulocyte, monocyte/macrophage adsorptive in pediatric ulcerative colitis. *World J Gastroenterol* 2016; 22(17): 4389-4396 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i17/4389.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i17.4389

INTRODUCTION

Active ulcerative colitis is associated with extravasation of large numbers of activated granulocytes and monocytes into the colonic mucosa. This infiltration is promoted by potent pro-inflammatory cytokines such as tumor necrosis factor-α, interleukin-8, leukotriene B4 and platelet-activating factor. Activated leukocytes can cause extensive mucosal tissue injury through the release of degradative proteases, reactive oxygen derivatives and pro-inflammatory cytokines[1]. While pharmacologic approaches target the inflammatory messengers, an alternative option reducing the activated cells and also reducing the associated circulating cytokines implicated in the pathogenesis of ulcerative colitis (UC) is selective granulocyte, monocyte/macrophage adsorptive (GMA) adsorptive using Adacolumn®, a medical device (JIMRO Co. Ltd., Takasaki-shi, Gunma, Japan). The selective adsorption of predominantly old and activated CD10+ neutrophils to Adacolumn carrier beads is governed by opsonins C3b/C3bi, Fcγ receptors and the leukocyte complement receptors, while lymphocytes are spared[2]. Flow cytometry analyses during apheresis sessions have shown an initial drop in peripheral neutrophils and the emergence of naïve CD10 neutrophils from the bone marrow, which represents a qualitative change within the circulating neutrophil population[3].

To date published meta-analyses and systematic reviews favor Adacolumn® GMA over control therapy for inducing remission and response at 12 wk in adult moderate active adult UC[4-7].

Pediatric onset UC is more complex, extensive and severe compared to adult UC, and has a high rate of steroid dependency[8,9]. As for the data on Adacolumn use in children to date, there are limited small scale investigations and case reports which point to good response especially in the treatment of corticosteroid-dependent and corticosteroid-resistant pediatric UC patients, with good treatment tolerance, mild side-effects, and a comparable rate of relapses as seen with drug treatments[10].

MATERIALS AND METHODS

For a considerable group of children with UC, treatment options are limited especially after failure of conventional treatment. On the background of a retrospective on clinical results in children treated with GMA apheresis[11], the ADAPT trial was designed to generate prospective cohort data in order to report efficacy and safety levels in moderate active pediatric UC patients when treated with Adacolumn.

GMA procedures

Enrolled patients underwent apheresis treatment using the Adacolumn® GMA apheresis device (JIMRO, Japan; EU Authorized Representative: Otsuka Pharmaceuticals

Table 1 ADAPT trial key inclusion criteria

Children and adolescents < 18 yr and with a body weight ⩾ 30 kg
Ulcerative colitis documented by clinical symptoms, endoscopic findings and histology since at least 3 mo prior to inclusion
Moderate active ulcerative colitis at baseline, defined as a PUCAI score between 35 and 64
Pancolitis or left-sided colitis
Receiving or having received one or more of the following medicinal products before screening:
Sulfasalazine, mesalamine and other 5-aminosalicylic acid agents for 4 wk or more with a stable dose for the last 2 wk
0.5 mg/kg per body weight with a maximum of 20 mg per day of prednisone with a stable dose for the last 2 wk, or
6-mercaptopurine or azathioprine for 12 wk or more with a stable dose for the last 4 wk

PUCAI: Pediatric ulcerative colitis activity index.

Table 2 ADAPT schedule of assessments

Visit	01	02	03	04	05	06	07	08	09	10
Day	-07	00	07	14	21	28				
Week	-1	0	1	2	3	4	6	7	8	12
Apheresis		▲	▲	▲	▲	▲	(▲)	(▲)	(▲)	
Physical examination	•									•
Endoscopy/ EAI		•[4]								(•)[5]
PUCAI	•	•	•	•	•	•	•	•	•	•
Clin. Chemistry	•	•		•		•	•[2]	•[2]	•[2]	•
ESR	•	•		•		•	•[2]	•[2]	•[2]	•
Urinalysis	•	•		•		•	•[2]	•[2]	•[2]	•
Fecal sample	•									
Coagulation	•					•	•[2]	•[2]	•[2]	•
Vital signs	•	•	•	•	•	•	•[1]	•[1]	•[1]	•
Concomitant medication	•	•	•	•	•	•	•	•	•	•
Adverse events	•[3]	•	•	•	•	•	•	•	•	•

[1]Only for patients with treatment; [2]Only for patients with last treatment; [3]Only if an endoscopy was performed at post screening; [4]The endoscopy should be done in the period between six weeks prior to screening and 5 d post screening visit; [5]The timeframe for the optional endoscopy is 7 d prior and 7 d after Visit 10. PUCAI: Pediatric ulcerative colitis activity index.

Europe Ltd, UK), which is approved for clinical use in EU (CE-marked) and in Japan. Adacolumn is an adsorptive type, single-use column filled with cellulose acetate beads of 2 mm in diameter. The carriers adsorb leukocytes, mainly activated granulocytes and monocytes from peripheral venous blood, passing from one antecubital vein through the column at a flow rate of 30 mL/min and returned to the contralateral antecubital vein. One apheresis treatment usually lasted 60 min, during which a total of 1.8 L blood was exposed to the carriers[12]. In the present trial, all patients received one weekly Adacolumn® apheresis over 5 consecutive weeks. The investigator could decide to add up to three additional treatments in weekly intervals at his discretion.

Table 3 Categories of pediatric ulcerative colitis activity index changes at week 12

Significant improvement	⩾ 35
Moderate improvement	⩾ 20
Small improvement	⩾ 10
No change	< 10

Trial design and efficacy assessment

This study used the PUCAI score[13] which encompasses abdominal pain, rectal bleeding, stool consistency, number of stools per 24 h, nocturnal stools and activity level; and the endoscopy activity index acc. to Rachmilewitz (EAI) comprising granulation, vascular pattern, vulnerability of mucosa and mucosal damage. Key inclusion criteria are listed in Table 1.

Steroid-resistance or -dependency, defined as inability to completely withdraw steroids without inducing a relapse or flare-up of the disease, was an exclusion criterion. The patients were not to have received a re-treatment of UC with drugs other than 5-ASA and derivatives, azathioprine and/or corticosteroids, *e.g.*, immunosuppressants and biologicals; or topical therapy for ulcerative colitis within the last 2 wk, or previous Adacolumn treatment.

At baseline, PUCAI was evaluated; flexible endoscopy (colonoscopy or sigmoidoscopy) for determination of EAI was performed, hematology and clinical chemistry tests were completed; vital signs, concomitant medication, and adverse events were recorded. During their treatment phase, patients were evaluated every week. Flexible endoscopy (colonoscopy or sigmoidoscopy) was performed at post screening unless there was one available from within 6 wk before and 5 d after screening. Endoscopy was optional at the evaluation visit in week 12 (Table 2).

The primary response variable was defined as the improvement in disease activity index (PUCAI) at week 12 (Visit 10) compared to Baseline (Visit 02). Key Secondary response variables were the proportion of significant improvement, moderate improvement, small improvement and no change as per PUCAI categories at week 12 (Table 3), the proportion of patients without disease activity (PUCAI < 10) at week 12, and the difference in EAI between week 12 and Baseline for those patients with pre- and post-treatment phase endoscopies available. Safety was assessed during the course of the clinical investigation by monitoring adverse events (AE), assessment of vital signs and collection of laboratory parameters.

Ethical considerations

This investigation was conducted in accordance with the Good Clinical Practice Guidelines according to CPMP/ICH/135/95, with the Declaration of Helsinki, ISO 14155:2003, and all relevant national guidelines. The Clinical Investigation Plan (CIP) was submitted

Table 4 Patient demography

Characteristic	mean ± SD [range]	
Age (yr)	13.5 ± 2.6 [8.1-17.8]	
Weight (kg)	47.7 ± 11.3 [31.0-72.2]	
Height (cm)	157.3 ± 12.3 [132.0-175.0]	
Duration of disease	3.1 ± 3.2 [0.2-14]	
Smoking status	No patient ever smoked	
Male	*n* = 13	52.0%
Female	*n* = 12	48.0%
Caucasian	*n* = 22	88.0%
Oriental (near East)	*n* = 2	8.0%
Other	*n* = 1	4.0%
Pancolitis	*n* = 18	72%
Left sided	*n* = 7	28%
Entry PUCAI score	mean = 42.6	median = 40
Entry EAI score (median)	mean = 7.0	median = 7.5

to the structured Institutional Review Boards (ethics committees) of each investigational center and a positive vote was obtained prior to start of the enrolment, in accordance with local law. Hence, written informed consent was obtained from all patients and/or their legal guardians or representatives prior to participation in the clinical investigation.

Statistical analysis

Based on the assumption that the standard deviation of the primary response variable (change in PUCAI) is 20, we aimed at including a sample of 50 patients to ensure that the precision of the estimated mean change in PUCAI is ± 5 at $P > 0.95$. Where appropriate, data are presented as the average (mean ± SD) values. For efficacy response variables, 95% confidence intervals were provided. If the confidence interval was above 0 (*i.e.*, the lower limit of the confidence interval was greater than 0), then Improvement in PUCAI was considered as statistically significant. For efficacy analyses based on the ITT set, last observation carried forward (LOCF imputational method) was used in case of missing data. For all other analyses, no imputation was done. All statistical analyses were carried out using SAS® Version 9.2 under Windows® Server 2008. Statistical review of the study was performed by a biomedical statistician.

Datasets analyzed in this investigation were the Safety set, the intent-to-treat (ITT) and the per-protocol (PP) datasets. The Safety set included all enrolled patients, in whom at least one treatment was initiated. The primary efficacy endpoint was based on the ITT population, which was defined as all enrolled patients who received at least one treatment and for whom there was at least one valid post-baseline PUCAI measurement. The PP analysis set was defined as the subset of the ITT population who received the full course of assigned treatment and for whom there were valid efficacy values at week 12. All results are presented for the ITT population unless otherwise stated.

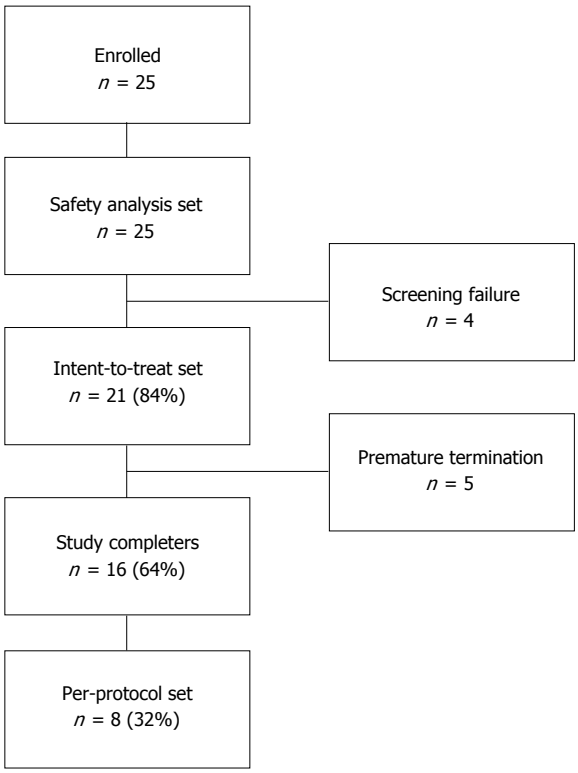

Figure 1 Patient disposition.

RESULTS

Patient demography

Twenty five children and adolescents with ulcerative colitis were enrolled (Figure 1, Table 4). All patients had at least one episode of active disease in the last 12 mo prior to enrollment in the clinical investigation.

Patient disposition

A total of 25 patients with moderate active UC (PUCAI score between 15 and 60) were screened and enrolled in the clinical investigation at 6 investigational centers. Twenty-five screened and enrolled patients entered the Safety Analysis set. There were four screening failures, one was due to detection of *Clostridium Difficile*, one was due to a diagnose change to Crohn's disease, and two patients with too low PUCAI scores were excluded from the trial. Twenty-one (84%) patients entered the ITT analysis set. Out of these, five patients prematurely terminated the clinical investigation due to adverse events (*n* = 2), intake of not permitted medication or physician's decision (*n* = 3). Sixteen patients (64%) completed the trial and 8 (32%) patients entered the Per-Protocol (PP) analysis set (Figure 1).

Concomitant medication

The most frequent used concomitant medication was Mesalazine, prescribed to 19 out of 25 patients (76%). Seven (7) of the 21 (33%) patients in ITT and 4 of the 8 (50%) patients in PP have used steroids during the clinical investigation. Fifteen out of 25 (60%) patients

Table 5 Improvement in PUCAI at week 12, patients with post-baseline scores

Analysis set	*n*	mean	SD	95%CI
ITT	20	22.3	19.9	12.9-31.6
PP	8	36.3	5.8	31.4-41.1

ITT: Intention-to-treat; PP: Per-protocol.

Table 6 Pediatric ulcerative colitis activity index category improvement, week 12 - intention-to-treat set

Category	*n*	%
Significant improvement	9	45.0
Moderate improvement	5	25.0
Small improvement	1	5.0
No change	5	25.0

Table 7 Changes in endoscopy activity index

Analysis set	mean	SD	95%CI	*n*
ITT	-3.0	2.5	-4.8-(-1.2)	10
PP	-2.8	2.5	-5.4-(-0.2)	6

ITT: Intention-to-treat; PP: Per-protocol.

have been prescribed immunosuppressants, 13 received Azathioprine, one patient Mercaptopurine and one patient Methotrexate.

Treatments administered

Nineteen out of 21 treated patients underwent at least 5 apheresis sessions, 17 patients received 6 treatments, 15 patients had 7 treatments, and 12 patients were treated with 8 Adacolumn aphereses.

Primary efficacy endpoint: The mean PUCAI improvement at week 12 was 22.3 (CI: 12.9-31.6) in the ITT population and 36.3 (CI 31.4-41.1) in PP analysis set (Table 5). Eight out of 15 patients (53%) in ITT and 4 out of 8 patients (50%) in PP were not on steroids. For these efficacy subsets, the PUCAI scores over time are depicted separately below (Figures 2 and 3).

Categorized PUCAI improvement: Defined as "significant improvement", "moderate improvement", "small improvement" and "no change" as per PUCAI categories at week 12 (Table 3), 70% of subjects had Significant improvement or moderate improvement; whereas a cumulated 30% of the patients experienced small improvement or no change comparing Visit 10 (week 12) *vs* baseline (Table 6).

EAI: At entry, the median EAI score was 7.5. Ten patients in the ITT dataset and six patients in the PP dataset had both endoscopies, at entry and at week 12. Calculated as per the available data, the mean change in EAI score was -3.0 for the ITT and -2.8 for the PP analysis set. Upper confidence intervals for both analysis sets were below zero (-1.2 for ITT and -0.2 for PP), indicating that EAI meaningfully decreased at week 12 compared to the screening visit (Table 7).

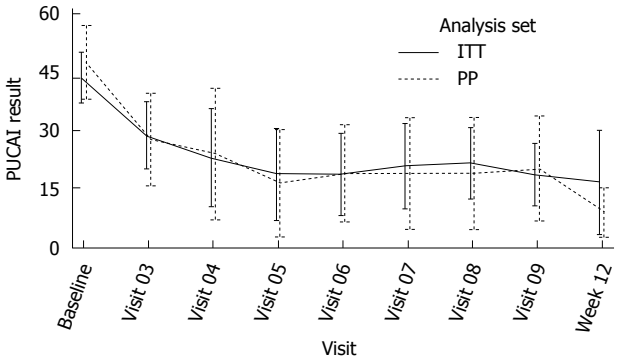

Figure 2 Pediatric ulcerative colitis activity index results over time by analysis set - Patients who received steroids.

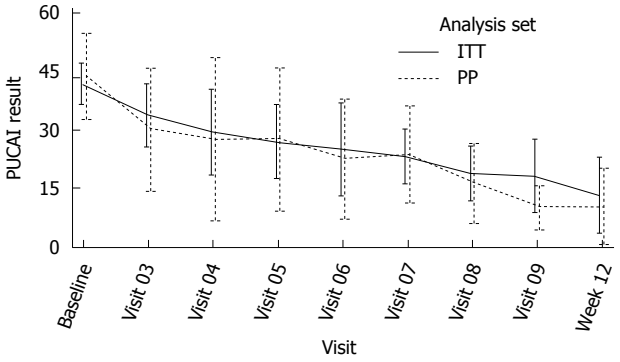

Figure 3 Pediatric ulcerative colitis activity index results over time by analysis set - Patients who did not receive steroids.

Disease activity: At Visit 03, Ulcerative Colitis disease activity of all patients (100%) was classified as "active". At week 12, 70% of the ITT and 50% of the PP analysis group were classified as active, reflecting a decrease in disease activity of 30% and 50% respectively.

High sensitive C - reactive protein (hsCRP): The mean change in hsCRP at Visit 10 was 0.6 for the ITT and -2.5 for the PP analysis set. The changes in hsCRP - levels were not significantly different between a specific visit and baseline visit in any direction. Similarly, no significant change was observed in hsCRP between baseline and the end of the follow-up phase in both ITT and PP analysis sets (mean changes were 0.9 and 0.5 mg/L respectively).

Further secondary endpoints: Seven (7) of the 21 (33%) patients in ITT and 4 of the 8 (50%) patients in PP have used steroids during the clinical investigation. The mean steroid dosage for steroid users in the ITT set from Baseline to Termination visit significantly decreased by 10.0 mg ($P < 0.04$)

Table 8 Adverse events, *n*

Relation	Severity	AEs; patients (safety set)
None	Mild	35; 12 (48%)
	Moderate	7; 4 (16%)
	Severe	0; 0 (0%)
	Total	42; 13 (52%)
Possibly or definitely related	Mild	15; 6 (24%)
	Moderate	6; 5 (20%)
	Severe	0; 0 (0%)
	Total	21; 8 (32%)

Clinical chemistry and hsCRP levels did not show any significant differences. Hematology, physical examination and vital signs did also not show any clinically significant changes throughout the clinical investigation.

Treatment safety and feasibility: During this clinical trial, up to week 12, no serious adverse event (SAE) occurred. Twenty one possibly or definitely related Adverse Events (AEs) were reported in 8 out of 25 (32%) patients, none of which was severe, 6 AEs were moderate, and 15 AEs were mild. Forty two unrelated AEs were furthermore recorded in 12 out of 25 (48%) patients. Unrelated mild transient headache, recorded for 6 patients, and procedural headache, recorded in 5 patients were the most prominent adverse events (Table 8).

Despite the additional challenges of venous access in pediatric patients, no more than 3 patients per visit experienced blood access problems, perfusions were stopped in no more than 4 patients per visit, and the flow rate was adjusted in no more than 4 patients per visit.

DISCUSSION

Therapeutic options in inflammatory bowel disease (IBD) continue to evolve. The Joint ECCO and ESPGHAN Evidence-based Consensus Guidelines aimed to develop guidelines for managing UC in children based on a systematic review (SR) of the literature and a robust consensus process of an international working group of specialists, also considering series on Adacolumn[10,11,14,15]. The overall number of pediatric UC patients in the literature is nevertheless still low and results confirm a persistent unmet medical need[16-19].

Infliximab is currently the only anti-TNFα approved in EU for pediatric UC patients for reducing signs and symptoms and inducing and maintaining clinical remission in moderately to severely active disease with a prior inadequate response to "conventional" therapy. In an overall pediatric UC cohort of 31 patients, the ratio of primary non-response to IFX was reported as 29% (9 out of 31 patients), and further 29% discontinued IFX after a median duration of treatment of 12.7 mo[17]. Data also lack for maintenance schemes with immunosuppressants alone or in combination with anti-TNFα[19].

The ADAPT trial endpoints and outcome parameters are in line with the recommendations published in the practical statement paper of the pediatric ECCO committee[8]. A limitation of our study is the low number of patients enrolled (n = 25): When designing the trial, the Standard Deviation estimate of the Primary Endpoint (mean change in PUCAI) was 20 points; hence a sample size of 50 subjects would have ensured that the precision of the estimated mean change in PUCAI is ± 5 (P > 0.95). ADAPT inclusion and exclusion criteria defined eligible patients to be on the one hand not treatment-naïve, and had on the other hand not (yet) steroid-resistant or steroid-dependent. Practically, this allowed only cases with ongoing steroid medication but not yet at the edge of treatment escalation or surgery. While this profile is not uncommon in adult UC, it turned out to be difficult to enroll pediatric patients, as there were fewer such patients than originally assumed, and their therapy is faster escalated nowadays.

Comparing the Safety to published results in pediatric and adult UC patients, there are two meta-analyses[4,5] and one systematic review[6]. The results all favor GMA apheresis over control therapy at week 12. Other groups (Tanaka *et al*[15]) communicated results from a series of 17 steroid-naïve consecutive pediatric UC patients over 5 years from a single center in Japan, having received Adacolumn treatment as monotherapy or in combination with low dose prednisolone after failure of first-line medication (sulphasalazine or mesalazine dosed at 2-4 g per day), and with a short duration of disease (median 6.5 mo). The group had used the adult CAI score. With 12 out of 17 patients responding to Adacolumn monotherapy in the Tanaka cohort, this reminds to some extent the subgroup of patients not having received steroids from our trial, and points to the favorable use of Adacolumn early in the course of the disease. The safety signals were transient mild headache in 8 patients, nausea and lightheadedness in 6 patients (35.3%), vomiting in 4 patients (23.5%). This compares quite well to the present ADAPT safety results; both as per profile and per occurrence rate. Looking to retrospective adult UC data as described in a large post-marketing surveillance study on GMA apheresis in 656 adult UC patients an overall positive outcome (remission or clinical response) was achieved in 77.3% of patients. The proportion of adverse effects in the adult population was only 2.3% (all mild and not requiring premature interruption of the procedure)[20].

The most common adverse event with Adacolumn GMA apheresis is headache, which is possibly due to transitory blood volume shifts while on extracorporeal circulation amounting to ca. 210 mL blood. The higher rate of transitory AEs like headache in the pediatric samples could hence be due to relatively

higher volume shifts and proportion of blood out in the extracorporeal system in pediatric patients, given their lower overall blood volume. On this background, it appears that the overall occurrence rate of adverse events is numerically less important in adult than in the pediatric patients of our cohort, but equal in nature and as mild and transient.

As for all induction treatments, transition to maintenance treatment and related compliance are a topic. Loss of response with or without antibody development seem to occur at least as often in pediatric patients as in adults, which is not the case with GMA maintenance schemes as published so far for adult UC patients: On-demand treatment with Adacolumn led to recurring remission, trend wise lasting longer than the prior remission phases[21].

Hematology and clinical chemistry tests did not show any treatment-related clinically significant changes throughout this clinical investigation, although the Adacolumn carriers deplete predominantly activated granulocytes and monocytes from the blood. The levels of these cells in the peripheral circulation are known to not be significantly lower after an apheresis session, which is due to a reactive influx of CD10 negative neutrophils from the bone marrow into the circulation ("pooling") within the first 20 min into an apheresis session[22].

Within the confidence interval boundaries calculated for the ADAPT trial at 25 patients, the outcomes in efficacy and safety levels at week 12 allow the assumption that Adacolumn treatment in a pediatric UC population yields comparable profiles of efficacy and safety as documented to date in adult UC treatment looking back on a decade of clinical experience.

In conclusion, GMA apheresis with Adacolumn® was safe and effective in pediatric patients with moderate active Ulcerative Colitis. The present data contribute to considering GMA apheresis as a therapeutic option in pediatric patients having failed first line therapy.

ACKNOWLEDGMENTS

Obituary: Sadly, Dr. Lena Grahnquist, Stockholm, passed away in January 2015 after long sickness. With the clinician's sharp eye, the researcher's intellect and her compassionate approach Lena Grahnquist combined the very best of what Pediatrics is about. We have lost a great person and colleague.

COMMENTS

Background

For a considerable group of children with ulcerative colitis (UC), treatment options are limited especially after failure of conventional treatment. The ADAPT trial was designed to generate prospective cohort data on efficacy and safety levels in moderate active pediatric UC patients when treated with Adacolumn granulocyte, monocyte/macrophage adsorptive (GMA) apheresis.

Research frontiers

Few therapeutic concepts in inflammatory bowel disease have a registered pediatric indication, and conducting clinical trials in children is particularly challenging.

Innovations and breakthroughs

The investigators report that the outcomes in efficacy and safety levels at week 12 allow the assumption that Adacolumn treatment in a pediatric UC population yields comparable profiles of efficacy and safety as documented to date in adult UC treatment looking back on a decade of clinical experience.

Applications

The present data contribute to considering GMA apheresis as a therapeutic option in pediatric patients having failed first line therapy.

Terminology

GMA apheresis is an extracorporeal, veno venous apheresis which selectively depletes neutrophils (granulocytes, monocytes) to adsorptive carriers in a single-use, sterile column. Adsorption to the carriers is governed by C3b/C3bi, FcgRs and the leukocyte complement receptors.

Peer-review

The authors aimed to investigate efficacy and safety of GMA prospectively in a population of pediatric patients with UC. In this study, significant improvement was detected in half of the patients who were treated. In adult patients with UC, surgery or anti TNF treatment might be considered. The present study suggests that Adacolumn treatment may be a useful option for pediatric patients in whom first line therapy has failed. Considering that GMA apheresis was well tolerated, this study provides useful new information.

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ORIGINAL ARTICLE

Observational Study

Clinical scenarios for the use of S100β as a marker of hepatic encephalopathy

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Abstract

AIM: To evaluate the association between serum concentrations of S100β in patients with cirrhosis and the presence of low grade hepatic encephalopathy (HE).

METHODS: This was a cross-sectional study. The population was categorized into four groups healthy subjects, cirrhosis without HE, cirrhosis with covert hepatic encephalopathy (CHE) and cirrhosis with overt HE. Kruskal-Wallis, Mann Whitney's *U* with Bonferroni adjustment Spearman correlations and area under the ROC were used as appropriate.

RESULTS: A total of 61 subjects were included, 46 cirrhotic patients and 15 healthy volunteers. S100β values were different among all groups, and differences remained significant between groups 1 and 2 ($P < 0.001$), and also between groups 2 and 3 ($P = 0.016$), but not between groups 3 and 4. In cirrhotic patients with HE S100β was higher than in

patients without HE [0.18 (0.14-0.28) ng/mL *vs* 0.11 (0.06-0.14) ng/mL, $P < 0.001$]. There was a close correlation between serum concentrations of S100β and psychometric hepatic encephalopathy score in patients with cirrhosis without HE compared to the patients with cirrhosis with CHE ($r = -0.413$, $P = 0.019$). ROC curve analysis yielded > 0.13 ng/mL as the best cutoff value of S100β for the diagnosis of HE (sensitivity 83.3%, specificity 63.6%).

CONCLUSION: Serum concentrations of S100β are higher in patients with cirrhosis than in healthy volunteers, and are further increased in the presence of hepatic encephalopathy. The results suggest that serum biomarkers such as S100β could help in the correct characterization of incipient stages of HE.

Key words: Hepatic encephalopathy; S100β protein; Astrocyte; Psychometric hepatic encephalopathy score; Critical flicker frequency

Core tip: Hepatic encephalopathy is a complication present in 30%-80% of the patients with cirrhosis and it is associated with increased mortality, adverse clinical outcomes, and poor quality of life. An increased concern about early recognition of this complication has risen in recent years; however, no biochemical marker is available to date. In this paper we evaluated the performance of S100β as a biochemical marker to identify the early stages of HE, and its correlation with neuropsychometric tests.

Duarte-Rojo A, Ruiz-Margáin A, Macias-Rodriguez RU, Cubero FJ, Estradas-Trujillo J, Muñoz-Fuentes RM, Torre A. Clinical scenarios for the use of S100β as a marker of hepatic encephalopathy. *World J Gastroenterol* 2016; 22(17): 4397-4402 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i17/4397.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i17.4397

INTRODUCTION

Hepatic encephalopathy (HE) reflects a spectrum of neuropsychiatric abnormalities seen in patients with liver dysfunction after exclusion of other brain diseases[1,2]. Covert hepatic encephalopathy (CHE) is the initial stage in the clinical spectrum of HE and involves subtle changes in cognitive and motor function, and electroencephalographic patterns. In contrast to patients with symptomatic HE, patients with CHE have no recognizable clinical symptoms of brain dysfunction and need to be diagnosed on the basis of neuropsychologic tests[3]. The prevalence of CHE in cirrhotics with good liver function (Child A) is 15%, while in those with advanced cirrhosis (Child B/C) it approaches 50%[4-6]. Multiple pathways are involved in the pathophysiology of HE, however, hyperammonemia plays a central role. Astroglial swelling in HE occurs if the load of glutamine accumulating during ammonia detoxification is not compensated by a decrease in other osmolytes like myo-inositol[7]. This is followed by development of low-grade cerebral edema, which is accompanied by an increased production of reactive oxygen and nitrogen species, triggering multiple protein and RNA modifications, thereby affecting brain function[8]. Associated abnormalities in the homeostasis of cerebral neurotransmitters and blood-brain barrier (BBB) disruption have not been well characterized yet. Different molecules have been addressed as markers of brain injury, some of them being linked to astrocyte damage, but to date there is no useful serologic marker that can aid the diagnosis of HE or CHE.

S100β is a 10.4 kDa protein that is primarily synthesized in the brain by the endfeet processes of the astrocytes, and it belongs to a superfamily of low molecular weight acidic calcium binding proteins of the EF-hand type[9]. This protein is primarily metabolized in the kidney and excreted in the urine, its expression across ethnic groups seems to be consistent, differences between genders are not found, and it does not seem to show circadian variation[10]. Although S100β is not entirely specific for the central nervous system (CNS), it is present in the brain at much higher concentrations than in other tissues (80%-90% of the total pool is found within the brain), and as such, this protein can be used as an early marker of brain damage[9].

Astrocytes are key in homeostasis regulation in the CNS, being activated after brain injury and releasing S100β[11]. Some studies have shown increased levels of S100β in patients with acute or chronic liver failure and HE which might reflect an early stage of intracerebral changes before the development of marked cerebral edema[12,13]. Moreover it has been proposed that increased serum concentrations of S100β could predict CHE[14]. However, evidence associating S100β levels and the presence of HE is scarce. The aim of this study was to evaluate the association between serum concentrations of S100β and the presence of low-grade HE in patients with cirrhosis.

MATERIALS AND METHODS

Patients

This was a cross-sectional analytic study. Healthy volunteers and ambulatory patients with cirrhosis were invited to participate. Cirrhosis was confirmed on the basis of liver biopsy, or presence of biochemical, ultrasonographic, and/or endoscopic features of portal hypertension and liver dysfunction. High-grade HE, use of psychoactive drugs, alcohol during the 3 mo prior to the start of the study, presence of renal failure, respiratory or central nervous system (CNS) disease, cardiac failure, or severe malnutrition

Table 1 Demographic characteristics of the study population

	Healthy subjects	C + non-HE	C + CHE	C + HE
n	**15**	**22**	**10**	**14**
Gender (M/F)	(8/7)	(11/11)	(6/4)	(9/5)
Age	51.5 (46-64.5)	53 (45-60)	65.5 (59-71.5)	66 (57-71)
Years of education	14 (6-19)	12 (6-16)	6 (4-9.7)	9 (6-16)
Main etiology (%)	--	HCV (34%)	HCV (40%)	HCV (66%)
MELD score	--	9.5 (8.5-11.5)	10 (7.7-15.2)	11.5 (10-17.5)
Child-Pugh (points)	--	7 (6-8)	7.5 (6-9)	10 (8-12)
Serum sodium	--	136 (129-143)	135 (126-139)	138 (122-141)
Ammonia	--	179 (113.5-224)	217 (194-250)	134 (95-277)
Total bilirubin	--	1.81 (1.11-2.48)	1.50 (1.26-2.58)	2.81 (2.09-5.94)
ALT	--	34 (28-55.2)	31 (24.5-95.5)	42.5 (32.2-65.5)
AST	--	62.5 (41-98)	49 (37.5-115)	77 (44-105.5)
Albumin	--	2.9 (2.6-3.3)	2.9 (2.6-3.2)	2.2 (2.0-2.7)
Alkaline phosphatase	--	141.5 (89.7-211)	140 (85-184)	154 (100.5-80.5)
INR	--	1.1 (1.1-1.2)	1 (0.9-1.2)	1.2 (1.1-1.7)
Creatinine	--	0.82 ± 0.19	1.11 ± 0.38	0.92 ± 0.31

Data presented as absolute frequencies, median (IQR) and mean ± SD. C: Cirrhosis; CHE: Covert hepatic encephalopathy; HE: Overt hepatic encephalopathy.

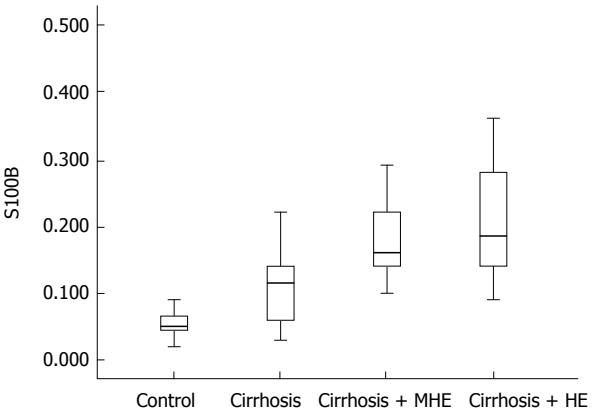

Figure 1 Serum concentrations of S100β (ng/mL) among groups. (1) Healthy subjects [0.05 (0.04-0.07)]; (2) Cirrhosis without HE [0.11 (0.06-0.14)]; (3) Cirrhosis with covert HE [0.16 (0.13-0.23)]; (4) Cirrhosis with overt HE [0.18 (0.14-0.28)]. Group 1 *vs* group 2, $P < 0.001$; Group 2 *vs* group 3, $P < 0.016$; Group 3 *vs* 4, $P = 0.508$. Healthy subjects *vs* all groups [0.14 (0.098-0.198)], $P < 0.001$.

were all considered exclusion criteria. History and physical examination, as well as psychometric hepatic encephalopathy score (PHES) test, and critical flicker frequency (CFF) analysis (Hepatonorm Analyzer; R&R Medi-Business Freiburg GMBH, Freiburg, Germany) were performed in all patients. Healthy volunteers did not show any neurologic abnormality on exam.

Methods

Overt HE was diagnosed on clinical grounds and classified according to West Haven criteria (all grade Ⅰ or Ⅱ). Covert HE (CHE)was identified in patients without overt manifestations of HE, when a PHES score was more than 4 SD below the Mexican population norms[15]. Using above criteria, 4 groups were formed: (1) healthy subjects; (2) cirrhosis without HE; (3) cirrhosis with CHE; and (4) cirrhosis with overt HE. Blood samples were drawn after an 8-h fasting period, and plasma collected and stored at -70 ℃ for further determination. S100β was measured using a human S100β enzyme-linked immunoassay (ELISA) according to manufacturer's instructions (BioVendor, Candler, United States). All tests were run in duplicate.

Statistical analysis

All results HE are expressed in medians and inter-quartile range, except for PHES, for which we used maximum and minimum values. The Kruskal-Wallis test was used to compare differences between groups for each of the variables of interest, while the *U* of Mann-Whitney with Bonferroni adjustment ($\alpha/\kappa = 0.016$) was used to evaluate differences between pairs of groups. Spearman test was used for bivariate correlations. ROC curve analysis was used to obtain a cutoff value for S100β. A P value < 0.05 was considered significant. SPSS version 21.0 was used for statistical analysis (IBM, Armonk, NY).

RESULTS

A total of 61 subjects were included, 46 cirrhotic patients and 15 healthy volunteers. The main characteristics of the population are shown in Table 1. Median age was higher in cirrhotics compared to the healthy subjects, most patients were males. The main etiology of cirrhosis was HCV and there was a stepwise increase in Child-Pugh and MELD score according to the presence and severity of HE.

The distribution of S100β serum values according from each group is shown in Figure 1. S100β values were different among all groups, and differences remained significant between groups 1 and 2 ($P < 0.001$), and also between groups 2 and 3 ($P = 0.016$),

Table 2 Psychometric evaluation

	Healthy subjects	C + non-HE	C + CHE	C + HE	*P* value
CFF	45.0 (43.0-48.6)	39.9 (39.2-41.4)	36 (33-39)	36 (35-40)	0.000
PHES	0 (-2 to 1)	-2 (-3 to -1)	-9 (-13 to -5)	-9.5 (-12 to –3)	0.000
Digit symbol	33.8 ± 18.3	28.91 ± 9.06	18.9 ± 8.8	13.4 ± 7.8	0.000
Number connection A	43.6 ± 21.1	49.5 ± 19.4	102.8 ± 66.5	148.8 ± 85.7	0.000
Number connection B	112.9 ± 39.1	147.2 ± 82.8	318.5 ± 153.03	435.7 ± 209.9	0.000
Serial dotting	62.3 ± 22.6	77.43 ± 18.1	127 ± 46.9	147.6 ± 64.3	0.000
Line tracing	95.2 ± 27.45	95.6 ± 28.6	193.8 ± 93.5	167.5 ± 37.8	0.000

Data presented as absolute frequencies, median (IQR) and mean ± SD. C: Cirrhosis; CHE: Covert hepatic encephalopathy; HE: Overt hepatic encephalopathy.

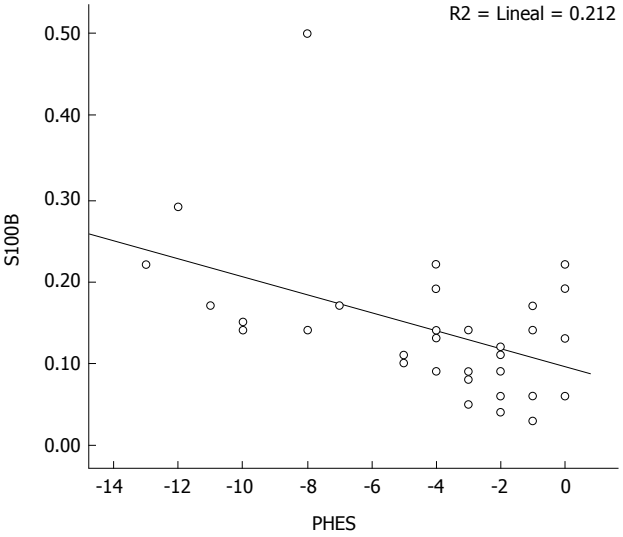

Figure 2 S100β and PHES correlations between patients with cirrhosis and covert HE and non-covert HE. *r* = -0.413, *P* = 0.019. HE: Hepatic encephalopathy; PHES: Psychometric hepatic encephalopathy score.

but not between groups 3 and 4. In cirrhotic patients with HE S100β was higher than in patients without HE [0.18 (0.14-0.28) ng/mL *vs* 0.11 (0.06-0.14) ng/mL, *P* < 0.001].

The results of the HE psychometric evaluation can be observed in Table 2. There was a clear deterioration in the performance of the CFF and PHES tests in the group with cirrhosis and cirrhosis with CHE or HE, as well as worsening in the individual PHES tests with a statistically significant difference.

In the total population, S100β was found to have a moderate correlation both with PHES (rho = -0.624, *P* < 0.001) and CFF (rho = -0.516, *P* < 0.001). In the subgroup of patients with cirrhosis, S100β showed moderate correlations with PHES (rho = -0.528, *P* < 0.001), as well as with CTP score (rho = -0.515, *P* < 0.001), and MELD score (rho = -0.440, *P* < 0.001), but not with CFF (rho = 0.240, *P* = 0.108) (Figure 2).

ROC curve analysis yielded > 0.13 ng/mL as the best cutoff value of S100β for the diagnosis of CHE and HE, with sensitivity of 83.3% and specificity of 63.6%. Area under the ROC curve (AUROC) was 0.801 (*P* < 0.0001).

DISCUSSION

HE and CHE are associated with decreased quality of life, impaired working performance, and patients become unfit to safely drive motor vehicles[16-21]. Cirrhotic patients with CHE are prone to develop episodes of overt HE when compared to those without CHE (incidence of 56% for those with CHE *vs* 8% for those without CHE at 3 years of follow up)[22]. Moreover, CHE is associated with a decreased survival, and thus it might be considered within the spectrum of complications definying advanced liver failure[23]. It is not known whether treatment of CHE may prevent overt HE or improve survival, but it is tempting to hypothesize that early identification of CHE followed by proper treatment should translate into improved quality of life, and have a favorable impact in prognosis. Psychometric tests are the preferred tool to diagnose CHE, and, since the final PHES score is adjusted to age and years of education, the differences of these two variables in our population did not have an impact in the results of our study.

S100β is not entirely specific for the CNS, but it is present in the brain at much higher concentrations than other tissues (80%-90% of the total pool is found within the brain), and as such, this protein can be used as a marker of brain damage[9]. Other places where S100β synthesis has been identified are adipose tissue[24], skin and melanoma tumors[25,26] and T-lymphocytes[27]. S100β protein is secreted by activated astrocytes and the mechanism of secretion remains unknown but astrocyte expression has been reported to be stimulated by interleukin-1 (IL-1) and by cyclic-AMP. The effects of secreted glial S100β depends on its concentration, being neurotrophic at low levels (nanomolar) and neurotoxic at high levels (micromolar)[9]. Nanomolar levels of S100β exert a stimulatory effect on astrocytes, causing glial proliferation *in vitro*[28]. S100β also accumulates in the extracellular space after astrocyte death or after cellular disintegration of the damaged parenchyma. Under these conditions, the S100β concentration may well be in the micromolar range and the protein may become toxic. This has originated the hypothesis that extracellular accumulation of S100β in ongoing brain

insults can, in combination with other unknown factors, cause a shift in its neuronal attributes from protective to hazardous[9].

We found significant differences in the serum concentrations of S100β between healthy volunteers and patients with cirrhosis. Moreover, in patients with cirrhosis, the levels of S100β increase in the presence of CHE and overt HE. The correlation between S100β and PHES indicates that at lower PHES scores, indicating a higher degree of HE, there is an increased concentration of S100β in serum. This suggests increased permeability of the BBB as the cognitive deterioration of HE progresses, what would determine not only leaking of S100β into the blood circulation, but increased ammonia delivery to the brain, leading to astrocyte activation and swelling. All of these changes (astrocyte activation and swelling, increased BBB permeability) seem to be present in patients with cirrhosis, and, moreover, seem to be more notorious in the presence of CHE and HE. However, our results would suggest that even in the absence of overt HE and CHE, the cirrhotic patient is at a state of astrocyte activation and swelling, and has increased BBB permeability. Brain adaptation to ammonia insults during early stages of cirrhosis and portal hypertension might consitute the counteracting condition preventing CHE or overt HE[29-31]. Our understanding of the cellular mechanisms leading to astrocyte dysfunction and neurotoxicity is limited, and it is unclear whether BBB disruption is crucial in the etiopathogenesis of HE, or an inevitable consequence of the disease itself. More studies are needed to better understand the role of S100β in the origin and perpetuation of HE in cirrhotic patients.

When we compared the cirrhotic patients without HE and CHE we found that S100β could differentiate both groups of patients with a fair accuracy, as shown by an AUROC curve of 0.801. Moreover, S100β correlated with the neuropsychometric tests (PHES and CFF), and with the hepatic functional status (MELD and CTP), attesting for its possible role in the evaluation of HE. Our results suggest that S100β can be useful in the diagnosis of HE, precisely in clinical scenarios where the diagnosis of HE remains unclear because behavior changes being subtle, evaluation of highly-educated patients, or when other psychiatric abnormalities (*i.e.*, depression) coexist or cannot be ruled out.

In conclusion, serum concentrations of S100β are higher in patients with cirrhosis when compared to healthy volunteers, and are further increased in the presence of CHE and overt HE. The results suggest the presence of astrocyte dysfunction, and maybe BBB disruption too, in patients with cirrhosis that seems to be more severe and progressive in the presence of HE. S100β showed significant differences between patients with cirrhosis but no encephalopathy at all, and the subgroup presenting with CHE, suggesting its usefulness to identify CHE. Although the AUROC curve was modest, S100β would potentially be useful in the diagnosis of low-grade HE whenever neuropsychometric tests are suboptimal, or when competing psychiatric differential diagnoses are in place.

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COMMENTS

Background

Hepatic encephalopathy (HE) is a frequent complication of cirrhosis, present in 30%-80% of patients. Covert HE has a profound impact in the quality of life of these patients, especially in daily functioning and driving ability, and it affects the sense of well-being. Thus, covert HE is very relevant to patients who suffer from it.

Research frontiers

The diagnosis and characterization of HE, especially in its low-grade forms, is difficult and requires specialized tests. Serum levels of ammonia were initially considered as a suitable marker of HE; however, they do not correlate with the degree of HE. Therefore, different serum markers for the diagnosis of HE, and particularly covert HE, are urgently needed.

Innovations and breakthroughs

Little attention has been paid to the use of biochemical markers as a means to detect early stages of HE. The data presented in the current study, indicate that S100β - a molecule released after astrocyte damage - might be a potential marker for covert HE in patients with cirrhosis. Interestingly, the association between high levels of S100β and presence of HE, as well as with the psychometric hepatic encephalopathy score, points towards a novel finding in the field.

Applications

The determination of S100β levels in serum, together with the standard clinical and psychometric evaluations, might help clinicians to establish the diagnosis of covert HE and therefore a timely treatment for these patients.

Terminology

S100β is a protein that is primarily synthesized in the brain by the processes of the astrocytes. Although S100β is not entirely specific to the central nervous system, it is present in the brain at much higher concentrations than in other tissues (80%-90% of the total pool is found within the brain), and as such, this protein could be used as an early marker of damage.

Peer-review

This manuscript mainly describes the association between serum concentrations of S100β and the presence of low-grade HE in patients with liver cirrhosis. The results suggest that S100β might help in the correct characterization of early stages of HE. The content is interesting and meaningful.

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ORIGINAL ARTICLE

Observational Study

Serum Mac-2 binding protein is a novel biomarker for chronic pancreatitis

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Informed consent statement: Written informed consent was obtained from all subjects at the time of enrollment or blood sampling.

Conflict-of-interest statement: The authors have no conflicts of interest to declare.

Data sharing statement: No additional data are available.

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Abstract

AIM: To determine the efficacy of Mac-2 binding protein (Mac-2bp) for diagnosis of chronic pancreatitis.

METHODS: Fifty-nine healthy volunteers (HV), 162 patients with chronic pancreatitis (CP), and 94 patients with pancreatic ductal adenocarcinoma (PDAC) were enrolled in this study. We measured serum Mac-2bp using our developed enzyme-linked immunosorbent assay kit. Additional biochemical variables were measured using an automated analyzer (including aminotransferase, alanine aminotransferase, γ-glutamyltransferase, alkaline phosphatase, triglyceride, C-reactive protein, and amylase levels) or chemiluminescent enzyme immunoassay (carbohydrate antigen 19-9 and carcinoembryonic antigen). The ability of Mac-2bp to predict CP diagnosis accurately was assessed using receiver operating characteristic (ROC) analyses.

RESULTS: Serum Mac-2bp levels were significantly increased in CP patients compared to HV ($P < 0.0001$) and PDAC patients ($P < 0.0001$). Area under the ROC curve values of Mac-2bp for the discrimination of CP from HV and PDAC were 0.727 and 0.784, respectively. Multivariate analyses demonstrated that serum Mac-2bp levels were independent determinants for CP diagnosis from HV and PDAC patients. Immunohistological staining showed that Mac-2bp was expressed faintly in the pancreas tissues of both CP and PDAC patients. Serum aspartate aminotransferase, alanine aminotransferase, γ-glutamyltransferase, alkaline phosphatase, and triglyceride levels were significantly higher in patients with CP or PDAC. Serum Mac-2bp levels were highly correlated with protein levels of alanine aminotransferase, γ-glutamyltransferase, and C-reactive protein, but not amylase, suggesting that the damaged liver produces Mac-2bp.

CONCLUSION: Measurement of serum Mac-2bp may be a novel and useful biomarker for CP diagnosis as well as liver fibrosis in the general population.

Key words: Pancreatic ductal adenocarcinoma; Chronic pancreatitis; Biomarker; Steatopancreatitis; Mac-2 binding protein (LGALS3BP)

Core tip: Serum Mac-2 binding protein (Mac-2bp) levels were significantly increased in chronic pancreatitis patients compared to healthy volunteers and pancreatic ductal adenocarcinoma patients. Therefore, measurement of serum Mac-2bp is a novel method for diagnosing chronic pancreatitis.

Maekawa T, Kamada Y, Ebisutani Y, Ueda M, Hata T, Kawamoto K, Takamatsu S, Mizutani K, Shimomura M, Sobajima T, Fujii H, Nakayama K, Nishino K, Yamada M, Kumada T, Ito T, Eguchi H, Nagano H, Miyoshi E. Serum Mac-2 binding protein is a novel biomarker for chronic pancreatitis. *World J Gastroenterol* 2016; 22(17): 4403-4410 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i17/4403.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i17.4403

INTRODUCTION

Chronic pancreatitis (CP) is characterized by progressive destruction of the pancreas tissue and recurrent episodes of intractable abdominal pain accompanied by exocrine and endocrine pancreatic insufficiencies[1,2]. CP is morphologically defined as progressive pancreatic fibrosis and inflammation that is accompanied by atrophy of pancreatic parenchymal cells[1,2]. CP is a strong risk factor for pancreatic ductal adenocarcinoma (PDAC) occurrence[3-5]. Recently, we investigated 76 PDAC patients who underwent surgery and analyzed pancreatic histological changes in specimens of noncancerous lesions[6]. Interestingly, we found that pancreatic steatosis, inflammation, and fibrosis in noncancerous lesions were each significant and independent determinants for PDAC. These histological changes are known as steatopancreatitis, and exactly the same characteristics are observed in the nonalcoholic steatohepatitis (NASH) liver. NASH, a severe form of nonalcoholic fatty liver disease (NAFLD), is a growing medical problem in industrialized countries around the world, and can progress to liver cirrhosis and hepatocellular carcinoma (HCC). We hypothesized that steatopancreatitis-induced CP would exist in the pathological background of PDAC comparable to NASH-related HCC occurrence.

PDAC is one of the most fatal cancers with a poor prognosis[7-11]. Most patients with PDAC are diagnosed at an advanced stage when the tumors are unresectable[7]. Even in the resectable cases, the prognosis of patients with PDAC is very poor because of local recurrence or distant metastasis occurring within a short period after the operation[9-11]. In our previous report, we found none of the 76 PDAC patients had a past history of clinical CP[6]. Therefore, the diagnosis of subclinical CP should be an important predictive factor for the early detection of PDAC. However, no useful diagnostic methods for the clinical diagnosis of CP have been identified.

Recently, we identified Mac-2 binding protein (Mac-2bp) as a novel diagnostic serum biomarker for NASH and fibrosis[12,13]. Mac-2bp is a glycoprotein that has

seven potential *N*-glycosylation sites[14,15]. Serum Mac-2bp concentrations increase in patients with breast and lung cancers, viral hepatitis, and autoimmune diseases[14]. Mac-2bp is rarely detectable in normal liver, but strongly detected in hepatocytes from chronic hepatitis type C (CHC) patients as liver fibrosis progresses[16]. Using proteome analysis, serum Mac-2bp is reported as a potential fibrosis marker in CHC patients[17]. In addition, *Wisteria floribunda agglutinin* (WFA)-positive Mac-2bp was recently reported as a novel serum fibrosis biomarker for CHC[18,19] and NASH[12,13,20]. Now, serum Mac-2bp is recognized as a novel and useful liver fibrosis biomarker in the clinic.

Therefore, we hypothesized that serum Mac-2bp also would be increased in CP patients with pancreatic fibrosis progression, and could be a useful biomarker for CP diagnosis. In this study, we measured serum Mac-2bp in 59 healthy volunteers (HV), 162 patients with CP, and 94 patients with PDAC, and investigated the availability of serum Mac-2bp as a CP diagnostic biomarker in comparison with other biochemical data.

MATERIALS AND METHODS

Ethics committee approval

This study was approved by the ethics committee of Osaka University Hospital (No. 260), and the study was conducted in accordance with the Helsinki Declaration. Written informed consent was obtained from all subjects at the time of enrollment or blood sampling.

Study subjects

Fifty-nine HV subjects were enrolled in this study from the aMs New Otani Clinic. We defined HV as the subjects who revealed no abnormal values in their laboratory evaluation without ultrasound-diagnosed fatty liver in health check-ups. One hundred sixty-two clinically diagnosed CP patients and 94 PDAC patients were enrolled in this study from Ogaki Municipal Hospital, Japan Community Health Care Organization Osaka Hospital, and Osaka University Hospital. The diagnosis of CP was made according to the guidelines of the Japan Pancreas Society[21]. The exclusion criteria from this study included a history of hepatic disease, such as chronic hepatitis C, chronic hepatitis B (seropositive for hepatitis B surface antigen), autoimmune hepatitis, Wilson's disease, or hepatic injury caused by substance abuse. Sera from these subjects were collected and frozen at -80 ℃ until use. Study subjects in this study were enrolled from 2002 to 2013.

Laboratory measurements and Mac-2bp ELISA

Serum biochemical variables [aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltransferase (GGT), alkaline phosphatase (ALP), albumin (Alb), total bilirubin (T-Bil), creatinine (Cr), total cholesterol (T-Chol), triglyceride (TG), C-reactive protein (CRP), amylase (AMY)] were measured with a conventional automated analyzer. Serum levels of carbohydrate antigen 19-9 (CA19-9) and carcinoembryonic antigen (CEA) were determined using a chemiluminescent enzyme immunoassay. We measured serum Mac-2bp using our developed ELISA kit (Immuno-Biological Laboratory Co., Ltd., Fujioka, Japan, code No. 27362) as previously reported[12].

Statistical analysis

Statistical analysis was conducted using JMP Pro 11.0 software (SAS Institute Inc., Cary, NC). Variables were expressed as the mean ± standard deviation (SD). Statistical analysis included descriptive statistics, analysis of variance, the Wilcoxon and Kruskal-Wallis tests, and Spearman R correlations. As Mac-2bp, AST, ALT, GGT, T-Bil, TG, AMY, CRP, CA19-9, and CEA did not show a Gaussian distribution, these parameters were common log-transformed before analysis. The diagnostic performances of the scoring systems were assessed by analyzing receiver operating characteristic (ROC) curves. The probabilities of true positive (sensitivity) and true negative (specificity) assessments were determined for selected cut-off values, and the area under the ROC curve (AUC) was calculated for each index. The Youden index was used to identify the optimal cut-off points. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Serum biochemical variables and Mac-2bp levels in study subjects

The descriptive characteristics of the study subjects are shown in Table 1. The average age and serum AST, ALT, GGT, ALP, TG, and CEA were significantly lower in HV subjects than in CP and PDAC patients. T-Chol, CRP, AMY, and platelet count were significantly higher in CP patients than in HV subjects and PDAC participants. Serum Alb was significantly lower and average age and CA19-9 were significantly higher in PDAC patients than in CP patients. Serum Mac-2bp levels were significantly higher in CP patients than in both HV subjects and PDAC patients (Table 1, Figure 1).

Relationships between serum Mac-2bp levels and other biochemical tests

The results of Pearson's correlations between serum Mac-2bp levels and other biochemical tests are summarized in Table 2. Mac-2bp levels showed significant positive correlations with AST, ALT, GGT, and CRP.

Ability of serum Mac-2bp levels to distinguish HV from CP patients

We investigated the ability of serum Mac-2bp levels to distinguish CP patients from HV subjects using the ROC curve (Figure 2A). The AUC value for Mac-2bp in distinguishing CP patients from HV subjects

Table 1 Clinical and serological characteristics of the subjects in this study

	HV	CP	PDAC	*P* value 1[1]	*P* value 2[2]
Number	59	162	94		
Age (yr)	48.2 ± 8.1	60.8 ± 14.5	66.7 ± 8.2	< 0.0001	< 0.05
Gender (F/M)	29/30	53/109	29/65	< 0.05	NS
AST (U/L)	18.4 ± 4.4	56.1 ± 246.0	39.2 ± 39.3	< 0.0001	NS
ALT (U/L)	15.3 ± 5.9	41.8 ± 156.7	42.9 ± 60.5	< 0.0005	NS
GGT (U/L)	28.0 ± 33.1	132.9 ± 317.0	98.8 ± 188.8	< 0.0001	NS
ALP (U/L)	179.5 ± 63.0	229.4 ± 92.6	325.8 ± 281.3	< 0.0001	NS
Alb (g/dL)	4.23 ± 0.22	4.18 ± 0.39	3.83 ± 0.37	NS	< 0.0005
T-Bil (mg/dL)	0.79 ± 0.29	0.88 ± 0.69	1.50 ± 3.26	NS	NS
Cr (mg/dL)	0.78 ± 0.19	0.77 ± 0.28	0.71 ± 0.19	NS	NS
T-Chol (mg/dL)	183.2 ± 26.5	202.7 ± 37.5	166.2 ± 33.0	< 0.05	< 0.0005
TG (mg/dL)	71.2 ± 25.6	120.3 ± 59.5	105.6 ± 48.0	< 0.0001	NS
CRP (mg/dL)	0.074 ± 0.119	1.98 ± 4.08	0.89 ± 2.09	< 0.0001	< 0.005
AMY (U/L)	71.5 ± 24.5	318.2 ± 1223.0	73.0 ± 44.8	< 0.0001	< 0.0001
Platelet (× 10^4/μL)	20.8 ± 4.7	23.9 ± 9.1	19.3 ± 6.7	< 0.05	< 0.005
CA19-9 (U/mL)	9.3 ± 7.9	38.7 ± 157.9	904.1 ± 3064.3	NS	< 0.0001
CEA (ng/mL)	1.35 ± 1.07	3.57 ± 4.72	7.31 ± 16.44	< 0.0005	NS
Mac-2bp (μg/mL)	1.33 ± 0.72	2.30 ± 1.75	1.32 ± 0.95	< 0.0001	< 0.0001

[1]*P* values (Wilcoxon test) correspond to the comparison between HV and CP; [2]*P* values correspond to the comparison between PDAC and CP. HV: Healthy volunteers; CP: Chronic pancreatitis patients; PDAC: Pancreatic ductal adenocarcinoma patients; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: γ-glutamyltransferase; ALP: Alkaline phosphatase; Alb: Albumin; T-Bil: Total bilirubin; Cr: Creatinine; T-Chol: Total cholesterol; TG: Triglyceride; CRP: C-reactive protein; AMY: Amylase; CEA: Carcinoembryonic antigen; Mac-2bp: Mac-2 binding protein; NS: Not significant.

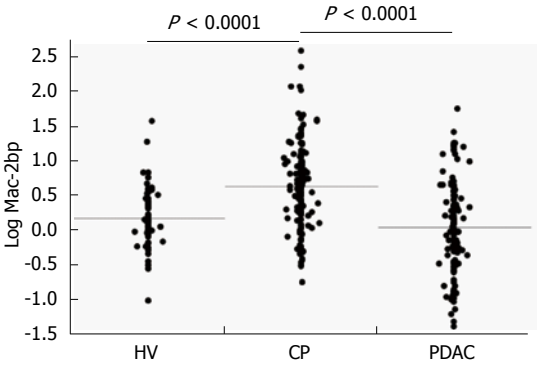

Figure 1 Serum Mac-2 binding protein levels were significantly elevated in chronic pancreatitis patients. Serum Mac-2 binding protein (Mac-2bp) levels in each group [healthy volunteer (HV), chronic pancreatitis patients (CP), pancreatic ductal adenocarcinoma patients (PDAC)]. Each dot on the graph is one individual. Horizontal gray lines indicate the mean values of common log-transformed Mac-2bp in each group.

was 0.727, and the cut-off value was 1.83 μg/mL with 87.9% sensitivity and 55.4% specificity. Multiple logistic analysis was performed to assess the CP diagnostic ability between HV subjects and CP patients (Table 3). Clinical variables that were significantly different between HV subjects and CP patients in univariate analysis were included except for ALT, which was strongly correlated with AST (Table 1). Age, TG, CRP, CEA, and Mac-2bp were significant independent variables for CP diagnosis. The odds ratio of Mac-2bp for the diagnosis of CP was 4.04.

Ability of serum Mac-2bp levels to distinguish CP patients from PDAC patients

We also investigated the ability of serum Mac-2bp levels to distinguish CP patients from PDAC patients

Table 2 Correlation coefficients for the relationships between Mac-2bp and various parameters

Factors	R	*P* value
Age (yr)	0.1	NS
AST (U/L)	0.22	< 0.0005
ALT (U/L)	0.15	< 0.05
GGT (U/L)	0.17	< 0.01
ALP (U/L)	0.12	NS
Alb (g/dL)	-0.051	NS
T-Bil (mg/dL)	0.14	NS
Cr (mg/dL)	0.035	NS
T-Chol (mg/dL)	-0.028	NS
TG (mg/dL)	0.16	NS
CRP (mg/dL)	0.24	< 0.0005
AMY (U/L)	-0.010	NS
Platelet (×10^4/μL)	0.067	NS
CA19-9 (U/mL)	0.068	NS
CEA (ng/mL)	0.024	NS

The following factors were common log-transformed: Mac-2bp, AST, ALT, GGT, T-Bil, TG, AMY, CRP, CA19-9, and CEA. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: γ-glutamyltransferase; ALP: Alkaline phosphatase; Alb: Albumin; T-Bil: Total bilirubin; Cr: Creatinine; T-Chol: Total cholesterol; TG: Triglyceride; CRP: C-reactive protein; AMY: Amylase; CEA: Carcinoembryonic antigen; Mac-2bp: Mac-2 binding protein; NS: Not significant.

using the ROC curve (Figure 2B). The AUC values for Mac-2bp in distinguishing CP patients from PDAC patients was 0.784, and the cut-off value was 1.41 μg/mL with 83.0% sensitivity and 66.0 % specificity. Multiple logistic regression analysis was also performed to assess the ability to distinguish between CP and PDAC patients (Table 4). Clinical variables that were significantly different between CP and PDAC patients in univariate analysis were included (Table 1). Alb, AMY, Platelet, CA19-9, and Mac-2bp were significant

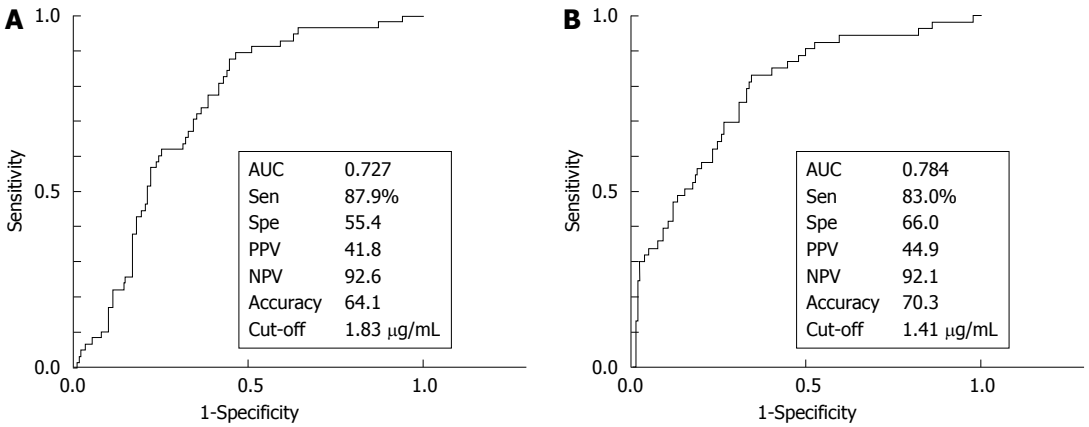

Figure 2 Serum Mac-2bp levels were useful for the discrimination of chronic pancreatitis patients from HV subjects and pancreatic ductal adenocarcinoma patients. A: ROC curve for the discrimination of chronic pancreatitis (CP) patients from HV subjects; B: ROC curve for the discrimination of CP patients from pancreatic ductal adenocarcinoma (PDAC) patients. AUC: Area under the curve; Sen: Sensitivity; Spe: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; Cut-off: Cut-off value.

Table 3 Multivariate analysis of factors associated with distinguishing between HV subjects and chronic pancreatitis patients

Factors	Odds ratio	95%CI	*P* value
Age	1.15	1.03-1.34	< 0.05
Sex (F/M)	2.30	0.25-27.57	NS
AST	1.16	0.93-1.58	NS
GGT	0.99	0.96-1.02	NS
ALP	0.98	0.95-1.01	NS
T-Chol	1.04	0.99-1.11	NS
TG	1.04	1.01-1.07	< 0.005
AMY	1.00	0.98-1.05	NS
CRP	229.6	3.30-5417218	< 0.005
CEA	2.31	1.35-4.92	< 0.05
Mac-2bp	4.04	1.82-11.06	< 0.05

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: γ-glutamyltransferase; ALP: Alkaline phosphatase; T-Bil: Total bilirubin; Cr: Creatinine; T-Chol: Total cholesterol; TG: Triglyceride; CRP: C-reactive protein; AMY: Amylase; CEA: Carcinoembryonic antigen; Mac-2bp: Mac-2 binding protein; NS: Not significant.

Table 4 Multivariate analysis of factors associated with distinguishing between PDAC and CP patients

Factors	Odds ratio	95%CI	*P* value
Age	0.60	0.09-1.25	NS
Alb	$3.76^{10} \times 11$	$922.3\text{-}1.31 \times 10^{45}$	< 0.0001
T-Chol	1.09	1.01-1.60	< 0.01
CRP	0.12	6.1×10^{-7} - 12.3	NS
AMY	1.32	1.05-2.69	< 0.005
Platelet	2.90	1.02-300.8	< 0.05
CA19-9	0.93	0.71-0.99	< 0.05
Mac-2bp	1.80	1.34-2.53	< 0.005

AMY: Amylase; T-Chol: Total cholesterol; CRP: C-reactive protein; Mac-2bp: Mac-2 binding protein.

independent variables for CP diagnosis.

DISCUSSION

In the present study, we found that serum Mac-2bp levels were increased in CP patients compared with in HV subjects and PDAC patients. Multivariate analyses demonstrated that Mac-2bp was an independent and significant determinant for CP discrimination from HV subjects and PDAC patients. Mac-2bp levels are known to increase in chronic liver diseases such as chronic hepatitis type C[18,19] and NASH[12,13,20]. Our results indicate that increased serum Mac-2bp in subjects without liver diseases should be evaluated for CP.

PDAC is one of the most fatal cancers with a poor prognosis, and most PDAC patients are diagnosed at advanced stages[7,9,10]. In advanced stages, PDAC is usually unresectable, and in even resectable cases, the prognosis of PDAC patients is very poor because of local recurrence or distant metastasis. Because CP is identified as a strong risk factor for PDAC occurrence[3-5], detection of CP in patients is important for the identification of early stage PDAC patients. Our present study indicates that the measurement of serum Mac-2bp is a useful strategy for detection of CP patients in the general population.

Very recently, we reported that a dramatic change in oligosaccharides on serum haptoglobin occurs between CP and PDAC patients[22]. In PDAC patients, we previously reported that serum fucosylated haptoglobin levels are significantly increased and could be a useful serum biomarker[23,24]. We measured serum total fucosylated haptoglobin and core-fucosylated haptoglobin by our developed lectin-antibody ELISA systems using *Aleuria aurantia* lectin (AAL), which recognizes all types of fucosylation, and *Pholiota squarrosa* lectin (PhoSL), which specifically recognizes core-fucosylation[25], respectively. Using these ELISA systems, we found serum core-fucosylated haptoglobin levels were significantly elevated in CP patients compared with HV subjects and PDAC patients[22]. Although the precise mechanisms through which serum core-fucosylated haptoglobin levels increase in CP patients are still unclear, these findings

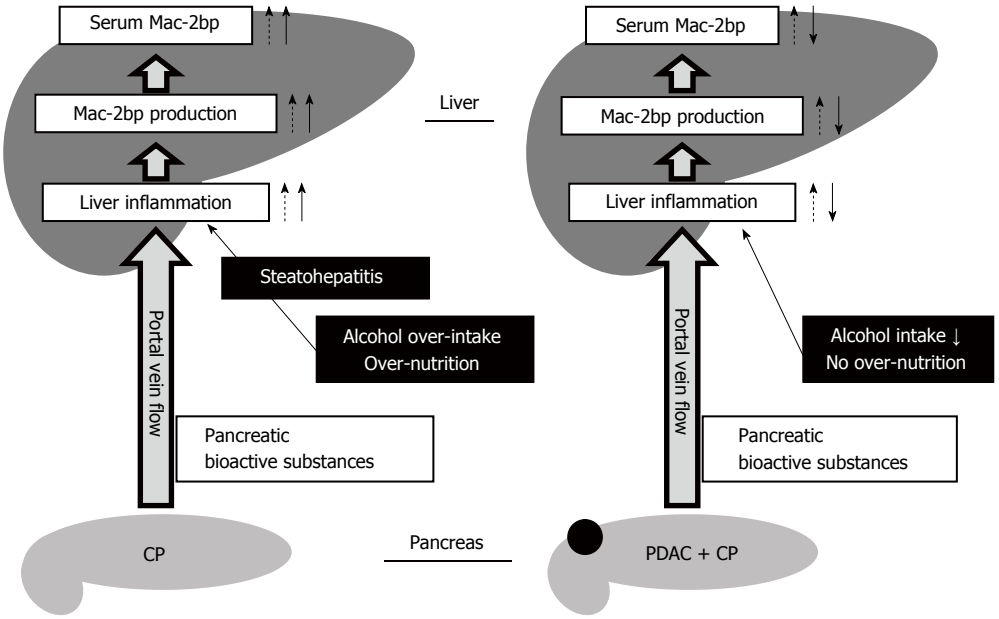

Figure 3 Putative mechanism of serum Mac-2bp changes in chronic pancreatitis and pancreatic ductal adenocarcinoma. In both chronic pancreatitis (CP) and pancreatic ductal adenocarcinoma (PDAC) patients, increased pancreatic bioactive substances produced in the pancreas flow into the liver through the portal vein and evoke inflammatory changes in the liver. This liver inflammation increases hepatic Mac-2bp production and serum Mac-2bp levels (Dotted arrows). Alcohol over-intake and/or relative over-nutrition induces steatohepatits in CP and further increases hepatic Mac-2bp production (left panel solid arrows). In contrast, the cessation of alcohol over-intake and no over-nutrition in PDAC would decrease hepatic Mac-2bp production and serum Mac-2bp levels would decrease in PDAC (right panel solid arrows).

indicate that core-fucosylation of glycoproteins would be enhanced in CP patients. Mac-2bp is a glycoprotein that we identified as one of the major fucosylated glycoproteins[12]. Therefore, using core-fucosylated Mac-2bp as a better biomarker is consistent with the findings of the present study.

To elucidate what cells produce Mac-2bp in the pancreas, we performed immunohistochemical staining for human Mac-2bp in PDAC and CP patients. However, Mac-2bp was expressed faintly in the pancreas tissues of both PDAC and CP patients (Supplementary Figure 1). These findings indicate that Mac-2bp was not produced in the pancreas. Previous reports demonstrate that tumor necrosis factor α and interferon γ can increase Mac-2bp expression in fibroblasts[26,27]. The blood stream carries abundant bioactive substances produced in the pancreas into the liver through the portal vein. Pancreatic inflammatory cytokines and cancer bioactive substances in CP and PDAC also flow into the liver and hepatic glycoprotein production, including Mac-2bp, would increase. Our previous reports demonstrate that serum Mac-2bp levels are significantly elevated in NASH patients compared to simple steatosis patients[12,13], and we found that serum Mac-2bp levels in CP patients were as high as those in NASH patients in the present study. Steatopancreatitis, induced by alcohol and/or energy over-intake, may promote the development of CP and is related to steatohepatitis[28-30]. Considering these findings, pancreatic bioactive substances would increase Mac-2bp production in the liver, and the presence of steatohepatitis under the condition of alcohol over-intake and/or relative over-nutrition further increases hepatic Mac-2bp production in CP patients. In contrast, no over-nutrition and cessation of alcohol over-intake in PDAC patients would contribute to the improvement of liver injury and might decrease the hepatic production of Mac-2bp (Figure 3). Indeed, our findings demonstrate that serum Mac-2bp levels were significantly and positively correlated with serum liver enzyme levels (AST, ALT, and GGT) and CRP (Table 2), but not with serum AMY levels. These results indicate that the liver may produce the elevated serum Mac-2bp in CP patients.

Our study has some limitations. Firstly, our CP and PDAC patients were clinically diagnosed with or without histological diagnosis. Therefore, the histological changes of noncancerous tissues of CP and PDAC patients were not fully assessed. In our future study, we would like to measure serum Mac-2bp levels in histologically diagnosed PDAC and CP patients. Secondly, age and gender among our study groups were significantly different. Ideally, age and gender would be matched among groups. However, the number of our study subjects was not enough to match these factors. Therefore, we performed multivariate analyses to adjust for these factors and found the significance of Mac-2bp even after adjusting for age and gender (Tables 3 and 4).

In conclusion, we find that serum Mac-2bp levels were significantly increased in CP patients compared with HV subjects and PDAC patients. This finding indicates that serum Mac-2bp can be used as a novel and useful CP biomarker. Measurement of serum Mac-2bp could

enable noninvasive screening for subclinical CP as well as chronic liver diseases in the general population.

COMMENTS

Background

Pancreatic ductal adenocarcinoma (PDAC) has the worst prognosis of all malignancies, and chronic pancreatitis (CP) is thought to be one of the main precursor diseases for PDAC occurrence. The authors recently found that inflammation and fibrosis are independent, characteristic histological changes in noncancerous lesions in PDAC patients despite the absence of a history of clinical CP. Therefore, the authors hypothesized that cryptogenic CP is an important predictive factor for PDAC occurrence. However, no useful biomarkers for the clinical diagnosis of CP have been identified.

Research frontiers

Recently, the authors identified Mac-2 binding protein (Mac-2bp) as a novel nonalcoholic steatohepatitis and fibrosis diagnostic serum biomarker. Mac-2bp is rarely detectable in normal liver, but strongly detected in hepatocytes from chronic hepatitis type C patients as liver fibrosis progresses. In the present study, we found that serum Mac-2bp levels were significantly increased in CP patients compared with healthy volunteers and PDAC patients. This finding indicates that serum Mac-2bp can be used as a CP biomarker.

Innovations and breakthroughs

In the present study, the authors found that serum Mac-2bp levels were increased in CP patients compared with in HV subjects and PDAC patients. Multivariate analyses demonstrated that Mac-2bp was an independent and significant determinant for CP discrimination from HV subjects and PDAC patients. Mac-2bp levels are known to increase in chronic liver diseases such as chronic hepatitis type C and NASH. The present results indicate that increased serum Mac-2bp in subjects without liver diseases should be evaluated for CP.

Applications

The present study found that serum Mac-2bp levels were significantly increased in CP patients compared with HV subjects and PDAC patients. This finding indicates that serum Mac-2bp can be used as a novel and useful CP biomarker. Measurement of serum Mac-2bp could enable noninvasive screening for subclinical CP as well as chronic liver diseases in the general population.

Peer-review

Very well written article, scientific content is important as conveying a new message using a novel biomarker for detection of subclinical Chronic pancreatitis. However the use of term subclinical CP in this study has been extrapolated to patient with CP as mentioned in methodology. This needs to be clarified whether these patients had any symptoms of CP or were detected incidentally from imaging findings. Also, author needs to define the term subclinical CP. Authors have also extrapolated their results in NASH patients but not mentioned whether these were same subjects with NASH and CP or PDAC, could they find a correlation?

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CASE REPORT

Subcapsular hepatic haematoma of the right lobe following endoscopic retrograde cholangiopancreatography: Case report and literature review

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Author contributions: Zappa MA, Aiolfi A and Porta A contributed to study concept/design, data collection, data analysis, data interpretation, writing the paper; Antonini I and Musolino CD contributed to data collection.

Institutional review board statement: This case report was exempt from the Institutional Review Board standards at University of Milan.

Informed consent statement: The patient involved in this study gave written informed consent authorizing use and disclosure of personal protected health information.

Conflict-of-interest statement: Marco Antonio Zappa, Alberto Aiolfi, Ilaria Antonini, Cinzia Domenica Musolino and Andrea Porta declare no conflicts of interests.

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Abstract

Sub capsular hepatic haematoma is a rare complication after endoscopic retrograde cholangiopancreatography (ERCP). Exact pathological mechanism is still unclear and few reports are nowadays available in literature. We report the case of a 58-year-old woman with recurrent episodes of upper abdominal pain, nausea and vomiting. On the basis of laboratory exams, abdomen ultrasound and magnetic resonance imaging she was diagnosed with a common bile duct stone. Endoscopic biliary sphincterotomy was performed. On the following day the patient complaint severe abdominal pain with rebound and hemodynamic instability. A computed tomography scan reveal a 14 cm × 6 cm × 19 cm sub-capsular hepatic haematoma on the right lobe that was successfully managed via percutaneous embolization. Sub capsular liver haematoma is a rare life threatening complication after ERCP that should be managed according to patients' haemodynamic and clinic.

Key words: Endoscopic guidewire; Endoscopic retrograde cholangiopancreatography; Abdominal pain; Subcapsular hepatic hematoma; Embolization

Core tip: Hepatic hematoma is a rare and potentially life threatening complication after endoscopic retrograde cholangiopancreatography (ERCP). Despite its severity, only few cases are described in current literature. The paper describe the management of a huge right lobe

hepatic hematoma following ERCP. An exhaustive literature analysis is made considering, signs and symptoms at presentation, time of presentation, diagnosis, and treatment. Awareness of this potential complication, high level of suspicion and prompt treatment are at the basis of better outcomes in such patients.

Zappa MA, Aiolfi A, Antonini I, Musolino CD, Porta A. Subcapsular hepatic haematoma of the right lobe following endoscopic retrograde cholangiopancreatography: Case report and literature review. *World J Gastroenterol* 2016; 22(17): 4411-4415 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i17/4411.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i17.4411

INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP) is a minimally invasive procedure for diagnosis and treatment of biliary and pancreatic disease. Complications occur in 2.5%-8% of cases with mortality rates ranging from 0.5%-1.0%[1]. Pancreatitis, cholangitis, perforation, and bleeding as a result of papillotomy are the most frequently described complications[2-3]. Sub capsular hepatic haematoma is a rare and potential life threatening condition[4]. We report the unusual case of a sub capsular hepatic haematoma after ERCP presenting with abdominal pain and hypotension.

CASE REPORT

A 58-year-old woman with recurrent episodes of upper abdominal pain was diagnosed with common bile duct stone by abdomen ultrasound and magnetic resonance imaging. She was admitted for ERCP and sphincterotomy. A proper drainage of the common bile duct was performed without complications. 12 h after the procedure the patient complaint a sudden abdominal pain with tenderness and rebound in the upper right quadrant without fever. Laboratory tests revealed a normal white blood cell count (7.44 × 10^9/L) and haemoglobin level (13.3 g/dL) with a slightly increased C-reactive protein (14.3 mg/dL). Total bilirubin, transaminases and amylases were within normal limits. Abdomen plain film was normal without signs of pneumoperitoneum. On the basis of such symptoms the patient was closely monitored. On the following 12 h she gradually develops hypotension (95/50 mmHg) and tachycardia (115 bpm) with a progressive haemoglobin decrease (8.6 g/dL). Urgent abdomen computed tomography (CT) scan demonstrated a large subcapsular hepatic haematoma of the right hepatic lobe supported by three peripheral parenchymal lacerations with contextual active bleeding and compression of the right and middle hepatic vein (Figure 1). On the basis of laboratory, clinical, and hemodynamic parameters the patient was urgently managed with percutaneous embolization of some small peripheral vessels on the sixth and seventh segment.

The post procedural course was uneventful with restoration of normal haemoglobin levels after transfusion (12.9 g/dL). Six days after embolization an abdomen CT scan shows the stability of the hematoma and the patient was discharged home.

DISCUSSION

Sub capsular hepatic haematoma is a rare and potentially life threatening complication after ERCP. Probably underestimated, only few cases are nowadays reported in literature and the exact pathological mechanism is unclear (Table 1). Accidental puncture of a peripheral intrahepatic biliar tree with consensual laceration of a small parenchymal vessels by endoscopic guide wire, may explain the phenomenon[2-4].

Sudden abdominal pain whenever associated with hypotension and tachycardia after ERCP should raise the suspicion of intrahepatic bleeding with Glisson's capsule distension. Different symptoms are described in literature: abdominal pain (91%), anemia (39.1%), hypotension (39.1%), fever (21.7%) and peritonism (13%) (Table 1). Laboratory tests did not provide major indicators of the development of a sub capsular hepatic haematoma, except for a decrease in the haemoglobin level[1]. Imaging modalities (ultrasound and CT) are the gold standard for diagnosis and surveillance of this emergent complication[9,15].

In the present case symptoms and signs started 12 h after the procedure with an early diagnosis and prompt treatment. Aspecific symptoms with a late onset from ERCP may occur with consequent delayed diagnosis and treatment (range 2-144 h) (Table 1).

Different treatment modalities are proposed in literature based on haemodynamic and clinics. The role of imaging in the assessment dimension and of ab extrinsic compression on hepatic vein is an important detail that should kept in mind whenever approaching such patients.

In stable patients with a limited, peripheral and non-compressive haematoma, a conservative management with prophylactic antibiotics should be suggested. Serial haemoglobin controls and abdomen CT verification is advisable[19]. Percutaneous drainage under CT guide and US should be proposed in case of abscess formation and fever[1].

Whenever hemodynamic instability is present with active bleeding and contrast extravasation, an immediate radiological or surgical approach should be taken into account. Minimally invasive radiological selective peripheral vessels embolization shows high success rates[21]. Surgical management should be reserved in case of general condition deterioration,

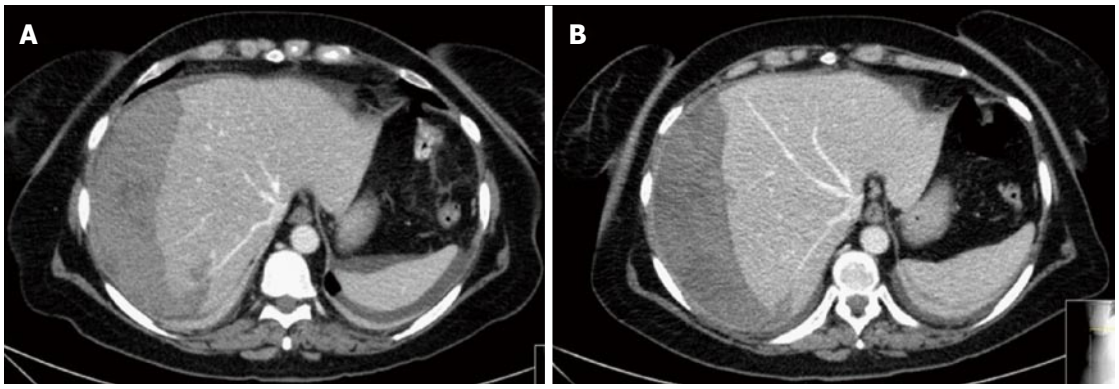

Figure 1 Urgent abdomen computed tomography scan. A: Hepatic subcapsular hematoma of the right lobe (14 cm × 6 cm × 19 cm) with peripheral parenchymal laceration. Ab-extrinsic compression of the right and middle hepatic vein with perisplenic free fluid; B: Six days after radiological selective embolization: note the stability of the haematoma dimension with disappearance of perisplenic free fluid.

Table 1 Subcapsular hepatic haematoma following endoscopic retrograde cholangiopancreatography: Review of the literature

Ref.	Indication for ERCP	ERCP	Onset of symptoms	Symptoms	Diagnosis	Dimension	Treatment	Death
Ortega Deballon *et al*[5]	Common bile duct stone	NA	NA	Abdominal pain	NA	NA	Percutaneous drainage	No
Horn *et al*[6]	Pancreatic adenocarcinoma	Cytologic brushing over a 0.035-inch guidewire + biliary stent	48 h	Abdominal pain/anemia	48 h; CT scan	NA (right lobe)	Conservative	No
Chi *et al*[7]	Pancreatic cancer	Biliary stent placement over a guidewire	NA	Abdominal pain	NA	NA	Embolization	No
Ertuğrul *et al*[8]	Hilar cholangiocarcinoma	Biliary stent placement over a guidewire	48 h	Abdominal pain/fever	48 h, CT scan	7.8 cm × 4.1 cm (right lobe)	Conservative	No
Priego *et al*[9]	Common bile duct stone	Spincterotomy over a guidewire	NA	Abdominal pain/ hypotension/ peritonism	NA, CT scan	4.7 cm × 10 cm × 11 cm (right lobe)	Surgery (Haematoma evacuation)	No
Petit-Laurent *et al*[10]	Common bile duct stone	Spincterotomy over a guidewire	48 h	Abdominal pain/fever	48 h; US/CT scan	NA	Percutaneous drainage	No
Bhati *et al*[11]	Common bile duct stone	Spincterotomy over a guidewire	NA	Abdominal pain/ hypotension	NA; CT scan	10 cm × 13 cm (right lobe)	Percutaneous drainage	No
Mc Arthur *et al*[12]	Common bile duct stone	Spincterotomy over a 0.035-ich guidewire + biliary stent	12 h	Abdominal pain/ leucocytosis	12 h; CT scan	5 cm × 3 cm (right lobe)	Conservative	No
De La Serna-Higuera *et al*[13]	Common bile duct stone	Spincterotomy over a 0.035-ich guidewire	48 h	Abdominal pain/ leucocytosis	72 h; abdomen US/ CT scan	14 cm × 8 cm × 5 cm (right lobe)	Conservative	No
Cárdenas *et al*[14]	bile leak after liver transplantation	Spincterotomy over a guidewire + biliary plastic stent positioning	24 h	Abdominal pain/anemia	NA, CT scan	NA	Conservative	No
Nari *et al*[15]	Acute biliary pancreatitis	NA	NA	Fever/ Abdominal pain	NA; CT scan	NA (right lobe)	Conservative	No
Revuelto Rey *et al*[16]	Common bile duct stone	Spincterotomy	6 h	Anemia	6 hours; CT scan	13 cm × 9 cm × 11 cm (right lobe)	Conservative	No
Baudet *et al*[17]	Common bile duct stone	Spincterotomy over a 0.035-ich guidewire	24 h	Abdominal pain/anemia/ fever/ hypotension	36 h; abdomen US/CT scan	16 cm × 6 cm, 5 cm × 21 cm (right lobe S6-7-8)	Embolization/ surgery (haematoma evacuation)	No
Pérez-Legaz *et al*[18]	Common bile duct stone	Spincterotomy	2 h	Abdominal pain/anemia/ hypotension/ peritonism	2 h; CT scan	8 cm (S5-6)	Surgery (electrocoagulation)	No
Del Pozo *et al*[19]	Common bile duct stone	Spincterotomy over a 0.035-ich guidewire	6 h	Abdominal pain	5 d; CT scan	NA, Right lobe	Conservative	No
Orellana *et al*[4]	Periampullary tumor	Biopsies + biliary plastic stent	4 h	Abdominal pain	4 h, CT scan	17 cm × 13 cm × 5 cm (right lobe)	Conservative	No

	Biliary stent occlusion	Stent exchange	2 h	Abdominal pain/ hypotension	2 h; CT scan	Hepatic hematoma covering the 50% of the total hepatic volume + hemoperitoneum	Embolization of the right epatic artery + peritoneal drainage under CT guidance under CT guidance	No
	Biliary stent disfunction in a patient affected by gallbladder cancer with consensual malignant biliary obstruction	Biliary plastic stent exchange	NA	Abdominal pain	NA, CT scan	Hepatic hematoma covering the 30% of the total hepatic volume	Conservative	No
Fei *et al*[1]	Common bile duct stone	Spincterotomy over a 0.035-ich guidewire	2 h	Fever	6 d; CT scan	13 cm × 6 cm (right lobe)	Percutaneous drainage	No
Klímová *et al*[20]	Wirsung stone	NA	6 h	Abdomial pain/anemia/ hypotension	NA	Right lobe	Embolization/ surgery/ percutaneous drainage	No
Zizzo *et al*[21]	Common bile duct stone	Spincterotomy over a 0.035-ich guidewire	24 h	Abdominal pain/ hypotension/ anemia	36 h; CT scan and angiography	15 cm × 11 cm (right lobe)	Embolization	No
González-López *et al*[22]	Iatrogenic benign stenosis following laparoscopic cholecistecotmy	Spincterotomy + Pneumatic dilation + biliary stent positioning	24 h	Abdominal pain/anemia/ hypotension/ peritonism	72 h; CT scan	NA (right lobe)	Surgery (damage control and packing)	Yes
Present case 2015	Common bile duct stone	Spincterotomy over a 0.035-ich guidewire	12 h	Abdominal pain/ hypotension/ anemia	24 h; CT scan	14 cm × 6 cm × 19 cm (right lobe)	Embolization	No

haemodynamic instability with signs of consensual peritoneal and free abdominal fluid[9]. Surgical approach consist in hematoma evacuation, local haemostasis with electrocoagulation or haemostatic devices, or packing in case of massive haemorrhage[22]. Literature data are in favour with a conservative treatment (43.5%), percutaneous embolization (26%), drainage (17.4%) and surgical management (13%) as a first line treatment. Failure of the first approach occur in 3 different cases (13%) without severe consequences (Table 1). Sudden rupture of the haematoma with consequent haemoperitoneum is a dreaded complications with high risk of mortality if misdiagnosed. González-López *et al*[22] report the case of a 30 years-old patient with Glisson's capsule rupture and consequent haemoperitoneum with consequent hypotension and signs of peritonism. The patients was surgically managed with electrocautery and packing without success.

Sub capsular liver haematoma is a rare and potentially life threatening complication following ERCP. Conservative treatment will be sufficient in most hemodinamically stable patients with no signs of super infection or abscess formation. Selective embolization is adequate in case of peripheral small vessels bleeding determining hemodynamic instability. Surgical approach is advisable in case of rupture risk, signs of peritonism and free abdominal fluid. Serial follow up CT scan are essential for dimension monitoring. We recommend that for legal purposes this potential risk should be addressed in the preoperative informed consent.

COMMENTS

Case characteristics

A 58-year-old woman with recurrent episodes of upper abdominal pain was diagnosed with common bile duct stone by abdomen ultrasound and magnetic resonance imaging and admitted for endoscopic retrograde cholangiopancreatography (ERCP) and sphincterotomy.

Clinical diagnosis

Hemodynamic instability, hypotension, and tachycardia were consistent with a post-procedural bleeding.

Differential diagnosis

Papillary bleeding after ERCP is one of the most common complications after the procedure. Splenic rupture, intrahepatic hematoma and visceral abdominal vessels rupture, related to instrumental looping with excessive traction, are exceptionally responsible for such situation.

Laboratory diagnosis

Laboratory tests did not provide major indicators in development of sub capsular hepatic haematoma.

Imaging diagnosis

Abdominal ultrasound and computed tomography (CT) scan are necessary for differential diagnosis.

Pathological diagnosis

A large sub capsular hepatic haematoma of the right lobe with active bleeding was evident on CT scan.

Treatment

On the basis of laboratory, clinical, and hemodynamic parameters the patient was urgently managed with percutaneous embolization of some small peripheral vessels.

Related reports

Probably underestimated, hepatic hematoma following ERCP is an extremely rare complication with few cases reported in current literature.

Experiences and lessons

Hepatic hematoma is a rare, potentially life threatening complication after ERCP. Awareness of such event is fundamental for early detection, diagnosis and treatment.

Peer-review

This report describe our experience in the management of a large hepatic hematoma after ERCP with an exhaustive literature review. Symptoms and signs at presentation, diagnosis, and management are reviewed in accordance to published literature. Limited number of literature reported cases is the major weakness of this study. Further studies are necessary to investigate the mechanism of injury and appropriate management of such complication.

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CASE REPORT

Inverted Meckel's diverticulum preoperatively diagnosed using double-balloon enteroscopy

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Author contributions: Takagaki K and Osawa S wrote the paper; Iwaizumi M, Hamaya Y, Furuta T and Sugimoto K contributed to the paper design and coordination; Takagaki K, Ito T and Osawa S performed the double-balloon enteroscopy; Wada H performed laparoscopic surgery; Tsukui H and Baba S contributed to the pathological examination.

Informed consent statement: The patient has provided permission to publish this paper, and the identity of the patient has been protected.

Conflict-of-interest statement: The authors declare no conflicts of interest associated with this manuscript.

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Abstract

An inverted Meckel's diverticulum is a rare gastrointestinal congenital anomaly that is difficult to diagnose prior to surgery and presents with anemia, abdominal pain, or intussusception. Here, we report the case of 57-year-old men with an inverted Meckel's diverticulum, who was preoperatively diagnosed using double-balloon enteroscopy. He had repeatedly experienced epigastric pain for 2 mo. Ultrasonography and computed tomography showed intestinal wall thickening in the pelvis. Double-balloon enteroscopy via the anal route was performed for further examination, which demonstrated an approximately 8-cm, sausage-shaped, submucosal tumor located approximately 80 cm proximal to the ileocecal valve. A small depressed erosion was observed at the tip of this lesion. Forceps biopsy revealed heterotopic gastric mucosa. Thus, the patient was diagnosed with an inverted Meckel's diverticulum, and single-incision laparoscopic surgery was performed. This case suggests that an inverted Meckel's diverticulum should be considered as a differential diagnosis for a submucosal tumor in the ileum. Balloon-assisted enteroscopy with forceps biopsy facilitate a precise diagnosis of this condition.

Key words: Inverted Meckel's diverticulum; Double-balloon enteroscopy; Small bowel tumor; Epigastric pain; Heterotopic gastric mucosa

Core tip: An inverted Meckel's diverticulum is a rare congenital anomaly of the gastrointestinal tract which is difficult to diagnose prior to surgery. This case report represents the utility of double-balloon enteroscopy for the precise preoperative diagnosis of an inverted Meckel's diverticulum.

Takagaki K, Osawa S, Ito T, Iwaizumi M, Hamaya Y, Tsukui H, Furuta T, Wada H, Baba S, Sugimoto K. Inverted Meckel's diverticulum preoperatively diagnosed using double-balloon enteroscopy. *World J Gastroenterol* 2016; 22(17): 4416-4420 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i17/4416.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i17.4416

INTRODUCTION

Meckel's diverticulum is a common asymptomatic congenital, gastrointestinal anomaly that is a remnant of the omphalomesenteric duct[1]. The incidence of Meckel's diverticulum was between 0.3%-4% in an autopsy series[1-3]. This anomaly may present as an inverted diverticulum causing anemia, abdominal pain, or intussusception[4]. Small bowel series and abdominal computed tomography (CT) scans have been used for the diagnosis of an inverted Meckel's diverticulum. However, the preoperative diagnosis of an inverted Meckel's diverticulum is difficult because the clinical and imaging features overlap with those of other etiologies or acute abdomen or gastrointestinal bleeding[5]. The final diagnosis is often made either intra-operatively or based on postoperative pathology reports. A recent advance in diagnostic methods involves balloon-assisted enteroscopy, which enables both the endoscopic observation of the entire small intestine as well as biopsy collection[6-8]. Here we report the rare case of a 57-year-old man with an inverted Meckel's diverticulum that was preoperatively diagnosed using double-balloon enteroscopy.

CASE REPORT

A 57-year-old man who had been repeatedly experiencing epigastric pain for 2 mo consulted at a clinic. He was then referred to another hospital due to a thickened intestinal wall observed on abdominal ultrasonography. His past medical history included depression and a gallbladder polyp treated by cholecystectomy. Physical examination and laboratory findings were unremarkable. An abdominal contrast-enhanced CT also showed a thickened small intestinal wall, with an elongated, intraluminal, fat-attenuating lesion in the lower abdomen (Figure 1). An upper gastrointestinal endoscopy and total colonoscopy were performed, but no abnormalities were found within the observed area. The patient was subsequently referred to our hospital for further examination. Retrograde double-balloon enteroscopy (DBE) was performed, which demonstrated an 8-cm, sausage-shaped, polypoid lesion that was located approximately 80 cm proximal to the ileocecal valve. A small depressed erosion was seen at the tip of the polypoid lesion, and intestinal villous mucosa was seen on the surface of the lesion (Figure 2). Using selective contrast-enhanced radiography with DBE, an elongated polypoid lesion was demonstrated in the ileum (Figure 3). Although Tc-99m pertechnetate scintigraphy was negative, a forceps biopsy from the tip of the depressed lesion revealed heterotopic gastric mucosa. The patient was diagnosed with an inverted Meckel's diverticulum, and he underwent single-incision laparoscopic surgery 3 mo later. An excavation of the serosa was observed at the basal region of the polypoid lesion by laparoscopy. After a Hutchinson's maneuver, segmental resection was performed. Pathological examination of the specimen revealed an 8.0 cm × 1.4 cm, sausage-shaped, polypoid lesion. Histological examination showed heterotopic gastric mucosa mimicking the pyloric glands at the tip of the lesion (Figure 4). The patient was discharged on post-operative day 6 without complications.

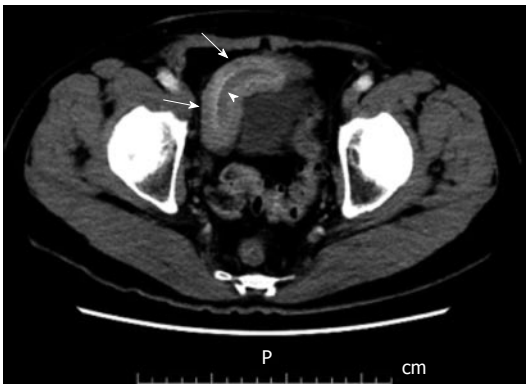

Figure 1 Contrast-enhanced computed tomography images of the lower abdomen. An abdominal computed tomography showed a thickened small intestinal wall (arrows) and an intraluminal, fat-attenuating lesion in the lower abdomen (arrowhead).

DISCUSSION

A Meckel's diverticulum is usually an asymptomatic condition, and many cases are incidentally discovered during a radiographic evaluation or during surgery performed for other reasons[1]. This condition causes symptoms in 4%-30% of patients[9], and possible complications include hemorrhage, bowel obstruction with or without intussusception, diverticulitis, and

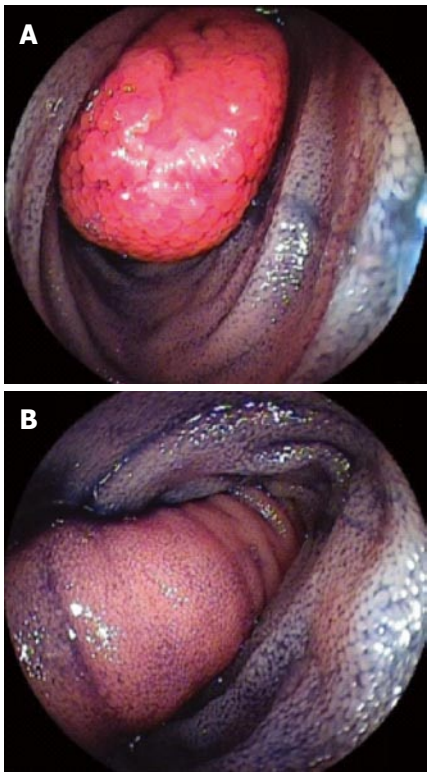

Figure 2 Double-balloon enteroscopy. A: Double-balloon enteroscopy revealed a sausage-shaped, elongated tumor with a depressed erosion at the tip in the ileum; B: Normal intestinal mucosa was seen on the surface of the tumor.

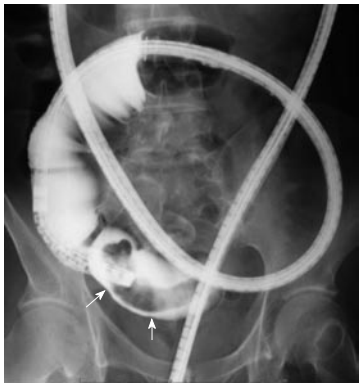

Figure 3 Selective contrast - enhanced radiography. An elongated, intraluminal polypoid lesion (arrows), approximately 8 cm in size, was seen in the terminal ileum.

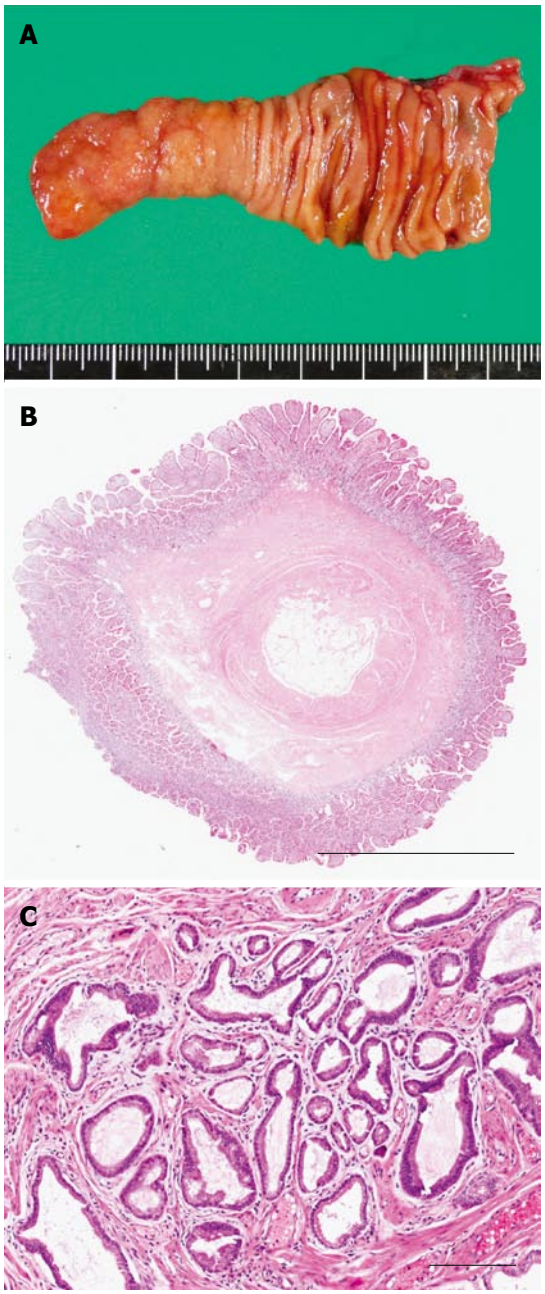

Figure 4 Pathological and histological examination. A: The opening of the specimen revealed an 8.0 cm × 1.4 cm polypoid lesion; B: Photomicrograph [hematoxylin and eosin (HE) stain] shows that the diverticulum was composed of all layers of the intestinal wall. Scale bars: 6 mm; C: Photomicrograph (HE stain) shows heterotopic gastric mucosa at the tip of the polypoid lesion. Scale bars: 600 μm.

inversion. According to a recent report, the most common complaint in an inverted Meckel's diverticulum is bleeding (80%), followed by anemia (78%) and abdominal pain (68%)[4]. The radiological features of an inverted Meckel's diverticulum are similar to that of a pedunculated intraluminal polyp, especially lipoma. Small bowel series reveal an elongated, smoothly margined, intraluminal mass that parallels the long axis of the intestine and frequently has a bulbous or club-sharped tip. Abdominal CT shows typical inverted cases as having a central core of fat attenuation[1].

This case seemed difficult to diagnose prior to surgery because the symptoms were mild and non-specific. Nevertheless, we could preoperatively diagnose the inverted Meckel's diverticulum using DBE. Recently, the reports of Meckel's diverticulum diagnosed using DBE are increasing[8]. DBE was developed by Yamamoto *et al*[6,7] in 2000 for the diagnosis and treatment of small intestinal disease. DBE is quite useful for diagnosis because it enables a biopsy of the entire small intestine[6]. Heterotopic gastric mucosa reportedly occurred in up to 50% of Meckel's diverticulum and up to 80% of symptomatic

patients[10]. Heterotopic pancreatic tissue was also found in 5%-16% of cases[1,11]. Meckel's diverticulum is typically located on the antimesenteric side of the distal ileum, within 20-100 cm proximal to the ileocecal valve[1]. Therefore, retrograde DBE is particularly recommended to diagnose Meckel's diverticulum[8,12].

The mechanism of inversion is not clearly understood. Levy *et al*[1] reported that 21% cases of Meckel's diverticulum were found to be inverted in the lumen of the small intestine. One theory is that abnormal peristaltic movement in proximity to the Meckel's diverticulum may cause it to invert[11,12]. Another theory is that because Meckel's diverticulum is not fixed to the mesentery or the intestine, it increases the likelihood of an inversion[4]. The mesenteric fat of the Meckel's diverticulum is pulled into the center of the diverticulum as it inverts in the small intestinal lumen. When the diverticulum is inverted, it may cause not only intestinal obstruction but also intussusception[1,4].

The preferred treatment for symptomatic Meckel's diverticulum is surgery. Whenever an inverted Meckel's diverticulum is diagnosed either pre-operatively or intra-operatively, the surgical procedure should be segmental resection. Laparoscopic resection is a safe and less invasive surgical procedure[1,4]. In this case, single-incision laparoscopic surgery was performed, which is a minimal access technique that can yield a reduction in scarring and consequently pain and suffering of the patients. Asymptomatic diverticula that are incidentally discovered remains controversial to date. The lifetime risk of developing complications is 4% up to the age of 20 years, 2% up to the age of 40 years, and 0% in the elderly population[1,4]. Therefore, preventive resection is recommended for patients younger than 40 years; for a diverticulum longer than 2 cm; diverticula with narrow necks, fibrous bands, or heterotopic gastric mucosa; or when the diverticula appears thickened and inflamed[4].

In the present case, an inverted Meckel's diverticulum with heterotopic gastric mucosa was clearly viewed using double-balloon enteroscopy. Therefore, an inverted Meckel's diverticulum should be considered as a differential diagnosis for a submucosal tumor in the ileum. Balloon-assisted enteroscopy combined with forceps biopsy enables a precise preoperative diagnosis.

COMMENTS

Case characteristics
A 57-year-old man had repeatedly experienced epigastric pain for 2 mo.

Clinical diagnosis
Physical examinations were unremarkable.

Differential diagnosis
Intestinal submucosal tumors: especially lipoma.

Laboratory diagnosis
All laboratory tests were within normal limits.

Imaging diagnosis
An abdominal contrast-enhanced computed tomography showed a thickened small intestinal wall, with an elongated, intraluminal, fat-attenuating lesion in the ileum, and retrograde double-balloon enteroscopy (DBE) demonstrated a sausage-shaped, polypoid lesion that was located approximately 80 cm proximal to the ileocecal valve. A small depressed erosion was seen at the tip of the polypoid lesion.

Pathological diagnosis
Histological examination showed that the diverticulum was composed of all layers of the intestinal wall, and heterotopic gastric mucosa was seen at the tip of the lesion.

Treatment
Single-incision laparoscopic surgery was performed.

Related reports
Although the reports of Meckel's diverticulum diagnosed using DBE are increasing, those of inverted situation is rare. DBE is quite useful for diagnosis.

Term explanation
An inverted Meckel's diverticulum is a condition where the Meckel's diverticulum literally inverts intraluminally on itself.

Experiences and lessons
An inverted Meckel's diverticulum is a rare congenital anomaly of the gastrointestinal tract which is difficult to diagnose prior to surgery. This case report represents the utility of double-balloon enteroscopy with biopsy for the precise preoperative diagnosis of an inverted Meckel's diverticulum.

Peer-review
The authors present a case report of inverted Meckel's diverticulum diagnosed pre-operatively. The report is well written.

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CASE REPORT

Spilled gallstones mimicking a retroperitoneal sarcoma following laparoscopic cholecystectomy

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Author contributions: Kim BS designed and wrote the paper; Joo SH supported writing of the paper; Kim HC reported radiologic imaging.

Institutional review board statement: This case report was approved by Kyung Hee University Hospital at Gangdong Institutional Review Board (KHNMC IRB 2015-12-008).

Informed consent statement: Informed consent was obtained from the patient for reporting this case.

Conflict-of-interest statement: All authors declare no conflicts of interest.

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Abstract

Laparoscopic cholecystectomy has become a standard treatment of symptomatic gallstone disease. Although spilled gallstones are considered harmless, unretrieved gallstones can result in intra-abdominal abscess. We report a case of abscess formation due to spilled gallstones after laparoscopic cholecystectomy mimicking a retroperitoneal sarcoma on radiologic imaging. A 59-year-old male with a surgical history of a laparoscopic cholecystectomy complicated by gallstones spillage presented with a 1 mo history of constant right-sided abdominal pain and tenderness. Computed tomography and magnetic resonance imaging demonstrated a retroperitoneal sarcoma at the sub-hepatic space. On open exploration a 5 cm × 5 cm retroperitoneal mass was excised. The mass contained purulent material and gallstones. Final pathology revealed abscess formation and foreign body granuloma. Vigilance concerning the possibility of lost gallstones during laparoscopic cholecystectomy is important. If possible, every spilled gallstone during surgery should be retrieved to prevent this rare complication.

Key words: Intra-abdominal abscess; Spilled gallstone; Laparoscopic cholecystectomy

Core tip: Gallstone abscess resulting from spilled gallstones is a rare complication following laparoscopic cholecystectomy. We report a rare presentation of gallstone abscess due to spilled gallstones following laparoscopic cholecystectomy, mimicking a retroperitoneal sarcoma in a 59-year-old male. Recognizing the patient information about a history of laparoscopic cholecystectomy and sharing the patient information with radiologists can make an accurate diagnosis and avoid misinterpretation when

the diagnosis is equivocal in radiologic imaging. Clear documentation of gallbladder perforation and gallstone can help with diagnosis of the complication and for correct management. Five noteworthy features are discussed in this paper.

Kim BS, Joo SH, Kim HC. Spilled gallstones mimicking a retroperitoneal sarcoma following laparoscopic cholecystectomy. *World J Gastroenterol* 2016; 22(17): 4421-4426 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i17/4421.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i17.4421

INTRODUCTION

Laparoscopic cholecystectomy is a standard treatment for symptomatic gallstone disease. Gallbladder perforation and gallstones spillage during laparoscopic cholecystectomy occurs in up to 40% of cases[1-3]. The incidence of unretrieved spilled gallstones has been estimated at 16% to 50%[3-5]. Gallstone abscess following spilled gallstones is an extremely rare delayed complication of laparoscopic cholecystectomy[3,6]. Common locations of the abscess are in the abdominal wall followed by intra-abdominal cavity usually in the sub-hepatic or retroperitoneum inferior to sub-hepatic space[3]. We report a rare case of abscess formation due to spilled gallstones mimicking a retroperitoneal sarcoma following laparoscopic cholecystectomy.

CASE REPORT

Our institutional review board approved the reporting of this case. A 59-year-old male was admitted to our clinic with a 1-mo history of worsening right-sided constant abdominal pain. Outside abdominal sonography showed a 4.5 cm mass-like lesion in the sub-hepatic space. He had no significant past medical history apart from a history of laparoscopic cholecystectomy about 5 mo previously. Laboratory workup revealed a white blood cell count of 7500/mm^3. All other laboratory findings including liver function tests, amylase and lipase were within normal limits. Tumor makers were normal. The hepatitis serologic markers were all negative. Computed tomography (CT) scan of the abdomen revealed a 4.9 cm × 4.6 cm right retroperitoneal mass invading the muscle wall and a 2 cm mass abutting the right kidney (Figure 1A-C). Magnetic resonance imaging (MRI) detected an ill-defined mass in the sub-hepatic space with subtle high signal intensity in T2 weighted image, low signal intensity in T1 weighted image and high signal intensity in diffusion weighted image (Figure 2A-C). With the suspicion that this mass was a retroperitoneal sarcoma, an exploratory laparotomy was performed. It revealed a severe adhesion around the mass containing purulent material and gallstones in the sub-hepatic space. Examination of frozen sections revealed acute and chronic inflammation with abscess and foreign body granuloma. Small abscess cavity found in the sub-phrenic space was destroyed using suction. Purulent materials were removed and several remaining stones were retrieved and copious irrigation was performed. Final pathology showed acute and chronic inflammation with abscess and foreign body granuloma (Figure 3). Postoperative recovery was uneventful. Six months later, the patient remains in good condition without any complaints.

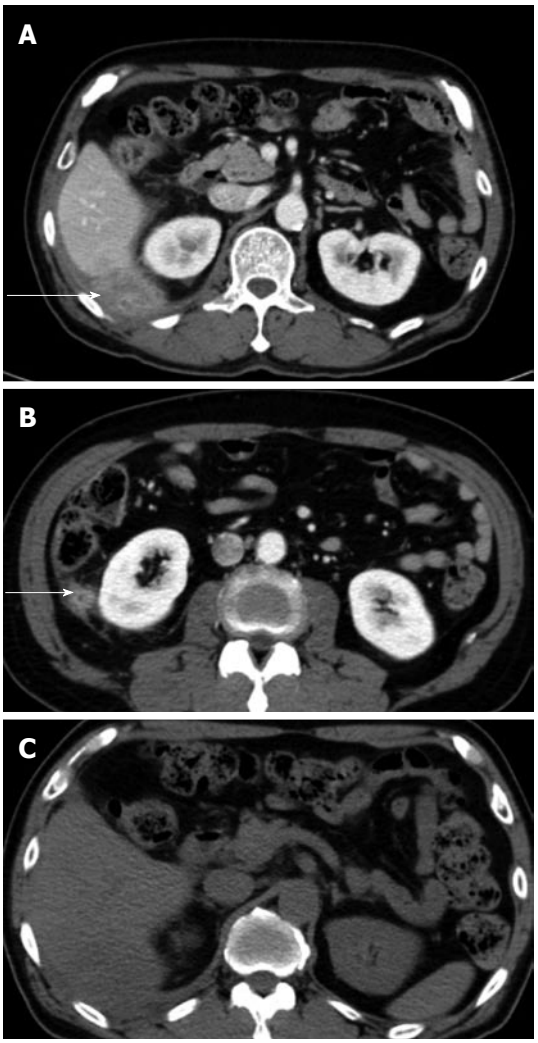

Figure 1 Five months after primary laparoscopic cholecystectomy, computed tomography scan was performed for evaluation of right upper quadrant pain. A and B: Abdominal CT scan revealed a 4.9 cm × 4.6 cm sized, ill-defined heterogenous retroperitoneal mass in posterolateral sub-hepatic and posterior perirenal space involving posterior abdominal wall (arrows); C: No calcified stone was seen to suggest spilled gallstones in the pre-contrast enhanced CT scan. CT: Computed tomography.

DISCUSSION

Gallbladder perforation and gallstone spillage during laparoscopic cholecystectomy occurs in up to 40% of cases[1-3]. The risk of gallbladder perforation leading to bile and gallstone spillage is more common during laparoscopic cholecystectomy than during

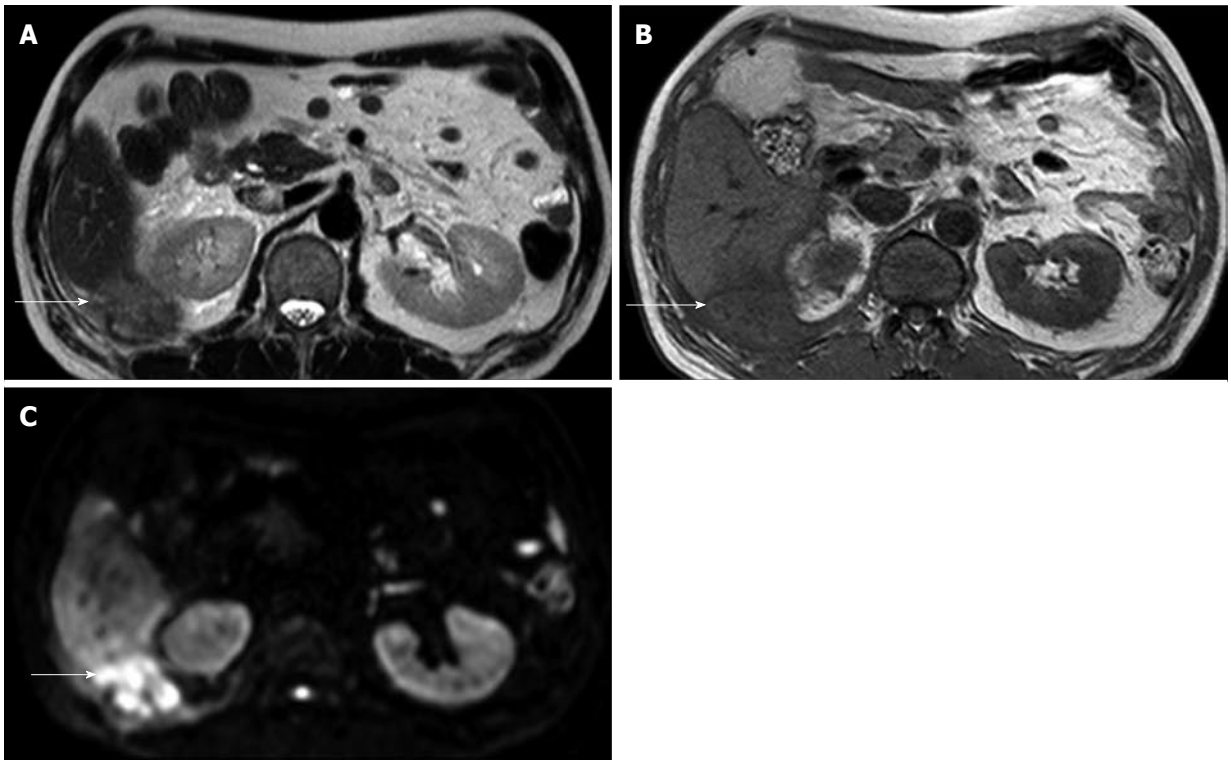

Figure 2 Magnetic resonance imaging showed ill-defined mass at right subhepatic space (arrows). A: Subtle high signal intensity in T2 weighted image; B: Low signal intensity in T1 weighted image; C: High signal intensity in diffusion weighted image.

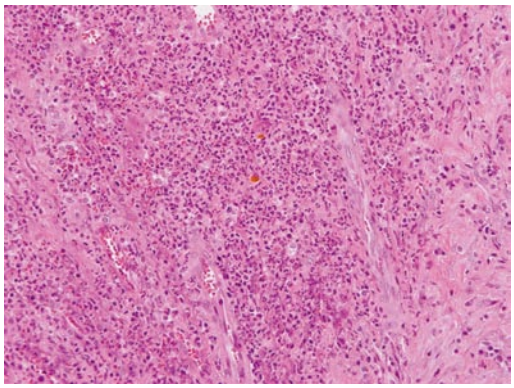

Figure 3 Numerous acute inflammatory cell and limphoplasma cell surrounding fibrosis and granulation tissue is seen. Bile stained gallstone sludge is seen in the center (haematoxylin and eosin staining, original magnification × 200).

open cholecystectomy. Gallbladder perforation most often occurs during the traction of the gallbladder or dissection from the gallbladder bed or extraction of the gallbladder through the port site, and it is more common in the presence of adhesions, inflammation around the gallbladder and in the early years of a surgical career[6,7]. The most important risk factor of gallbladder perforation is acute inflammation of the gallbladder with an edematous, friable or gangrenous wall[7,8]. However, gallstones spill into the abdominal cavity in only 7.3% of these cases[9,10].

Zehenter *et al*[10] reported all possible complications due to spilled gallstones during laparoscopic cholecystectomy. The most common complication is intra-abdominal abscesses that are located most often in the sub-hepatic space or its retroperitoneal region, as occurred presently. Fistula formations, hernia sacs, ovary and fallopian tubes containing lost gallstones are among some of the rare complications reported for gallstone abscess. These complications are rarely fatal. Thus, converting to an open procedure is not indicated, because only 8.5% of patients will lead to a complication[10].

An inflammatory response can be induced, which features walling off by omentum and local fibrosis. Inflammation and infection are more common with pigment stones[11]. This is postulated to be due to the release of bacteria from within the stones as the body breaks down the stone matrix[12]. In our case, preoperative CT in the primary procedure showed emphysematous cholecystitis with multiple stones (Figure 4). Operatively, gallbladder empyema with a friable wall was found (Figure 5). The edematous and friable wall hindered grasping of the gallbladder during the traction and the dissection of the gallbladder from the liver, which lead to gallbladder perforation and gallstones and infected bile spillage. Despite every effort to remove remaining stones including extensive peritoneal lavage and aspiration with a large suction tube (Figure 6), gallstone abscess due to spilled stones occurred 5 mo after laparoscopic cholecystectomy. This highlights that care must be taken not to spread gallstones into more inaccessible sites, making retrieval even more difficult. Spilled gallstones can be

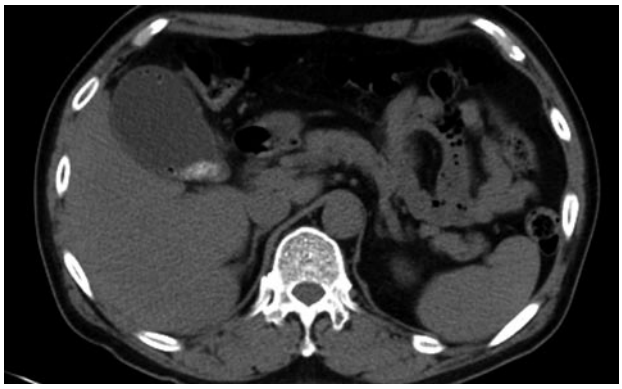

Figure 4 Computed tomography scan showing emphysematous gallbladder and gallbladder stones.

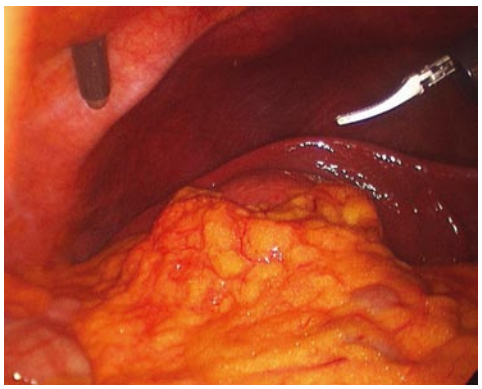

Figure 5 Operative findings in the primary procedure. Due to edematous, friable wall and gallbladder distension, it was hard to grasp the gallbladder during the traction and the dissection of the gallbladder from the liver.

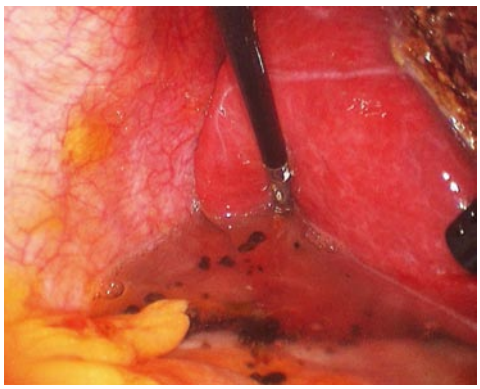

Figure 6 Multiple spilled gallstones. Copious irrigation of Morrison's pouch was applied.

retrieved easily in open cholecystectomy by irrigating the abdominal cavity and aspirating with a large suction tube or by collecting the stones with a sponge, which is difficult in laparoscopic cholecystectomy[13-15].

CT and MRI features of gallstones often include the presence of gallstones within the abscess, which is essential for a diagnosis[16]. Pigment stones with high calcium content are easily diagnosed with CT, whereas pure cholesterol stones and those with low calcium content may go undetected, as in our case[17]. Presently, CT and MRI were equivocal with the findings of retroperitoneal sarcoma. Spilled stones were not evident in radiologic imaging. Presence of the highly reflective echoes with posterior shadowing in the abscess cavity can be pathognomonic for gallstone abscess in patients with a history of laparoscopic cholecystectomy.

Abscess formation secondary to spilled stones mimicking a retroperitoneal sarcoma is rare. Despite their extreme rarity, the characteristic appearance of intra-abdominal abscesses from gallstones should be recognized because their radiographic appearance can mimic more severe disease, such as peritoneal metastasis[16,18]. Abscess formation from spilled stones after laparoscopic cholecystectomy has been reported to have an average duration of 4 mo to 10 years[19,20]. Presently, despite the history of laparoscopic cholecystectomy 5 mo before, gallstone abscess mimicking a retroperitoneal sarcoma in radiologic finding made the diagnosis difficult. Spilled gallstones around the liver may be confused with peritoneal metastasis or lymph nodes, having a similar appearance of round soft-tissue perihepatic nodules with a high attenuation peripheral rim on CT. Unenhanced CT may be useful in elucidating the calcified nature of these nodules. However, although the presence of calcification favours the diagnosis of spilled gallstones, some mucin producing tumours, such as tumours from the ovary and colon, can contain calcium[21]. Therefore, spilled gallstones should be considered in a patient with a history of LC presenting with an abdominal abscess.

The current case highlights five noteworthy features. Gallstone abscess resulting from spilled gallstones is a rare complication following laparoscopic cholecystectomy, but spilled gallstones should always be considered in a patient with a history of laparoscopic cholecystectomy presenting with an abdominal abscess. Secondly, a surgeon needs to share the patient information with radiologists to make an accurate diagnosis and avoid misinterpretation when the diagnosis is equivocal in radiologic imagings. Thirdly, careful manipulation of gallbladder with empyema, wall thickening and many gallstones during laparoscopic cholecystectomy should be made. Fourth, every effort to retrieve all spilled stones during laparoscopic cholecystectomy should be made to avoid late complications and the peritoneum should be irrigated with copious saline. Finally, it is important for the surgeon to clearly document in the operative record whether the gallbladder was perforated and whether stones spilled during laparoscopic cholecystectomy to help with diagnosis of the complication and for correct management.

In conclusion, retroperitoneal abscess formation secondary to spilled gallstones following laparoscopic cholecystectomy is a rare condition. If possible, every spilled gallstone during laparoscopic cholecystectomy

should be retrieved to prevent such a rare complication. As in our case, preoperative radiological investigations may be equivocal. Clear documentation of gallbladder perforation and gallstone spillage is important, as is being vigilant to the possibility of stones during surgery.

COMMENTS

Case characteristics

A 59-year-old male with a history of laparoscopic cholecystectomy 5 mo prior presented with a 1-mo history of worsening right-sided constant abdominal pain.

Clinical diagnosis

The patient presented with a 1-mo history of worsening right-sided constant abdominal pain.

Differential diagnosis

Retroperitoneal sarcoma, metastatic cancer.

Laboratory diagnosis

All labs were within normal limits.

Imaging diagnosis

Computed tomography showed a 4.9 cm × 4.6 cm right retroperitoneal mass invading the muscle wall and a 2 cm mass abutting the right kidney.

Pathological diagnosis

Acute and chronic inflammation with abscess and foreign body granuloma.

Treatment

Abscess cavity was destroyed using suction. Purulent materials were removed and several remaining stones were retrieved and copious irrigation was performed.

Related reports

Intra-abdominal abscesses secondary to spilled gallstones are located most often in the sub-hepatic space or its retroperitoneal region. Fistula formations, hernia sacs, ovary and fallopian tubes containing lost gallstones are among some of the rare complications reported for gallstone abscess.

Term explanation

The characteristic appearance of intra-abdominal abscesses from gallstones should be recognized because their radiographic appearance can mimic more severe disease, such as peritoneal metastasis and retroperitoneal sarcoma.

Experiences and lessons

Retroperitoneal abscess formation due to spilled gallstones following laparoscopic cholecystectomy is a rare condition. If possible, every spilled gallstone during laparoscopic cholecystectomy should be retrieved to prevent such a rare complication. Clear documentation of gallbladder perforation and gallstone spillage is important, as is being vigilant to the possibility of stones during surgery.

Peer-review

This case report is very useful to doctors in clinical practice. It is helpful to remind doctors to avoid the same mistake in clinical work.

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TOPIC HIGHLIGHT

2016 Hepatitis C virus: Global view

Thrombin activation and liver inflammation in advanced hepatitis C virus infection

Emilio González-Reimers, Geraldine Quintero-Platt, Candelaria Martín-González, Onán Pérez-Hernández, Lucía Romero-Acevedo, Francisco Santolaria-Fernández

Emilio González-Reimers, Geraldine Quintero-Platt, Candelaria Martín-González, Onán Pérez-Hernández, Lucía Romero-Acevedo, Francisco Santolaria-Fernández, Servicio de Medicina Interna, Hospital Universitario de Canarias, Universidad de La Laguna, Tenerife, 38320 Canary Islands, Spain

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Abstract

Hepatitis C virus (HCV) infection is associated with increased thrombotic risk. Several mechanisms are involved including direct endothelial damage by the HCV virus, with activation of tissue factor, altered fibrinolysis and increased platelet aggregation and activation. In advanced stages, chronic HCV infection may evolve to liver cirrhosis, a condition in which alterations in the portal microcirculation may also ultimately lead to thrombin activation, platelet aggregation, and clot formation. Therefore in advanced HCV liver disease there is an increased prevalence of thrombotic phenomena in portal vein radicles. Increased thrombin formation may activate hepatic stellate cells and promote liver fibrosis. In addition, ischemic changes derived from vascular occlusion by microthrombi favor the so called parenchymal extinction, a process that promotes collapse of hepatocytes and the formation of gross fibrous tracts. These reasons may explain why advanced HCV infection may evolve more rapidly to end-stage liver disease than other forms of cirrhosis.

Key words: Coagulation; Liver cirrhosis; Hepatitis C virus; Fibrogenesis; Parenchymal extinction; Portal thrombosis; Protein C

Core tip: Liver cirrhosis may be considered a pro-thrombotic condition despite it being associated with a low platelet count and deranged synthesis of clotting factors. When hepatitis C virus (HCV) is the etiological factor of liver cirrhosis, intrahepatic coagulation may

be enhanced by several direct actions of HCV on the clotting system, platelet aggregation, and altered anticoagulation. The excessive thrombin generation may be related to increased fibrogenesis both by a direct effect of thrombin on hepatic stellate cells and fibrosis related to ischemic parenchymal extinction.

González-Reimers E, Quintero-Platt G, Martín-Gonzále C, Pérez-Hernández O, Romero-Acevedo L, Santolaria-Fernández F. Thrombin activation and liver inflammation in advanced hepatitis C virus infection. *World J Gastroenterol* 2016; 22(18): 4427-4437 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i18/4427.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i18.4427

INTRODUCTION

Liver damage in hepatitis C virus (HCV) infection includes a wide range of clinical entities. Chronic infection may lead to chronic hepatitis, with more or less marked steatosis and/or steatohepatitis, cirrhosis, and hepatocarcinoma. In advanced stages of HCV infection, recent research has pointed out the importance of thrombotic events and microcirculatory changes within the liver. As in other forms of cirrhosis, but probably with a higher prevalence, in HCV-infected patients thrombin formation with accompanying microthrombosis of portal vein radicles takes place, a phenomenon that may accelerate the natural history of the disease. The factors involved in this process, and the clinical relevance of this prothrombotic situation are reviewed in the present work.

HEMOSTATIC SYSTEM

Coagulant and anticoagulant pathways: A schematic overview

Hemostasis, in its wide sense, involves adhesion, aggregation, and activation of platelets (primary hemostasis), and the formation of a fibrin clot as the final product of an autocatalytic process initiated by the damaged endothelium. As shown in Figure 1, thrombin, a protein derived from the action of activated factor Ⅹ on prothrombin, plays a central role. Thrombin promotes platelet activation and aggregation, and activates factor XI, factor Ⅷ and factor Ⅴ, creating a positive feed-back loop that underscores the need of a counter-regulatory system. This is composed by antithrombin, tissue factor pathway inhibitor, thrombomodulin, liver synthesized vitamin-K dependent protein C and its endothelial receptor, and protein S. Thrombomodulin is a transmembrane protein located on endothelial cells, in intimate connection with endothelial protein C receptor (Figure 2). Thrombomodulin acts as a thrombin receptor. Once thrombin and protein C bind to thrombomodulin and protein C receptor, protein C becomes quickly activated by thrombin, a process highly dependent on thrombomodulin. Once activated, protein C binds to protein S. This complex inhibits activated factor Ⅴ and factor Ⅷ, leading to decreased thrombin generation (Figure 2). Thrombin generation is also quenched by the presence of antithrombin, a protease with many actions, also synthesized in the liver, and tissue factor pathway inhibitor (Figure 1), whose levels are not decreased, but even increased in cirrhosis[1]. Antithrombin strongly inhibits thrombin, but also inhibits the activated forms of factors XII, XI, Ⅹ, Ⅸ and Ⅶ; plasmin and kallikrein, trypsin and C1[2-4]. Tissue factor pathway inhibitor forms a complex with factor Xa, inactivating it; and also blocking the tissue factor-factor Ⅶa complex. Like thrombomodulin, tissue factor inhibitor is an endothelial product[5] and, as discussed later, both may become altered in hepatitis C-dependent endothelial damage.

Platelets constitute another key component of hemostasis. Endothelial disruption exposes subendothelial collagen to the bloodstream, and this is followed by an avid binding of collagen to collagen-specific Ia/Ⅱa platelet surface receptors. Another endothelial-derived multimeric protein, von Willebrand factor, firmly interacts with platelet glycoproteins Ib/Ⅸ/Ⅴ and collagen fibrils. The function of this multimeric protein is controlled by a metalloprotease (ADAMTS 13), synthesized by several cells, including hepatic stellate cells and endothelial cells, among others[6]. Binding of collagen to glycoprotein Ⅵ receptor activate the platelets - a process also facilitated by thrombin which activates platelets by binding to other types of receptors, namely specific G protein receptors. Activated platelets release several products contained in their granules, including potent proinflammatory mediators, and finally aggregate and form a hemostatic plug. Aggregated platelets efficiently anchor fibrin, forming a plug that stops bleeding. Platelet membrane phospholipids bind to the gamma carboxy residues of activated factors Ⅸ and Ⅹ, serving as a "platform" on which coagulation takes place. Calcium mediates this binding.

In order to avoid excessive thrombus formation, fibrinolysis is activated. This is also a tightly regulated process, by which an inactive protein - plasminogen - transforms into an active one - plasmin, which is able to destroy formed fibrin. A series of substances, such as tissue plasminogen activator, factor XIIa, and urokinase plasminogen activator transform plasminogen into plasmin, whereas several others block this effect. Indeed, plasminogen activator inhibitor, thrombin activatable fibrinolysis inhibitor (TAFI)[7], plasmin inhibitor, and histidine rich glycoprotein are all potent antifibrinolytic agents.

Thrombosis in HCV infection

There is considerable evidence that the hepatitis C

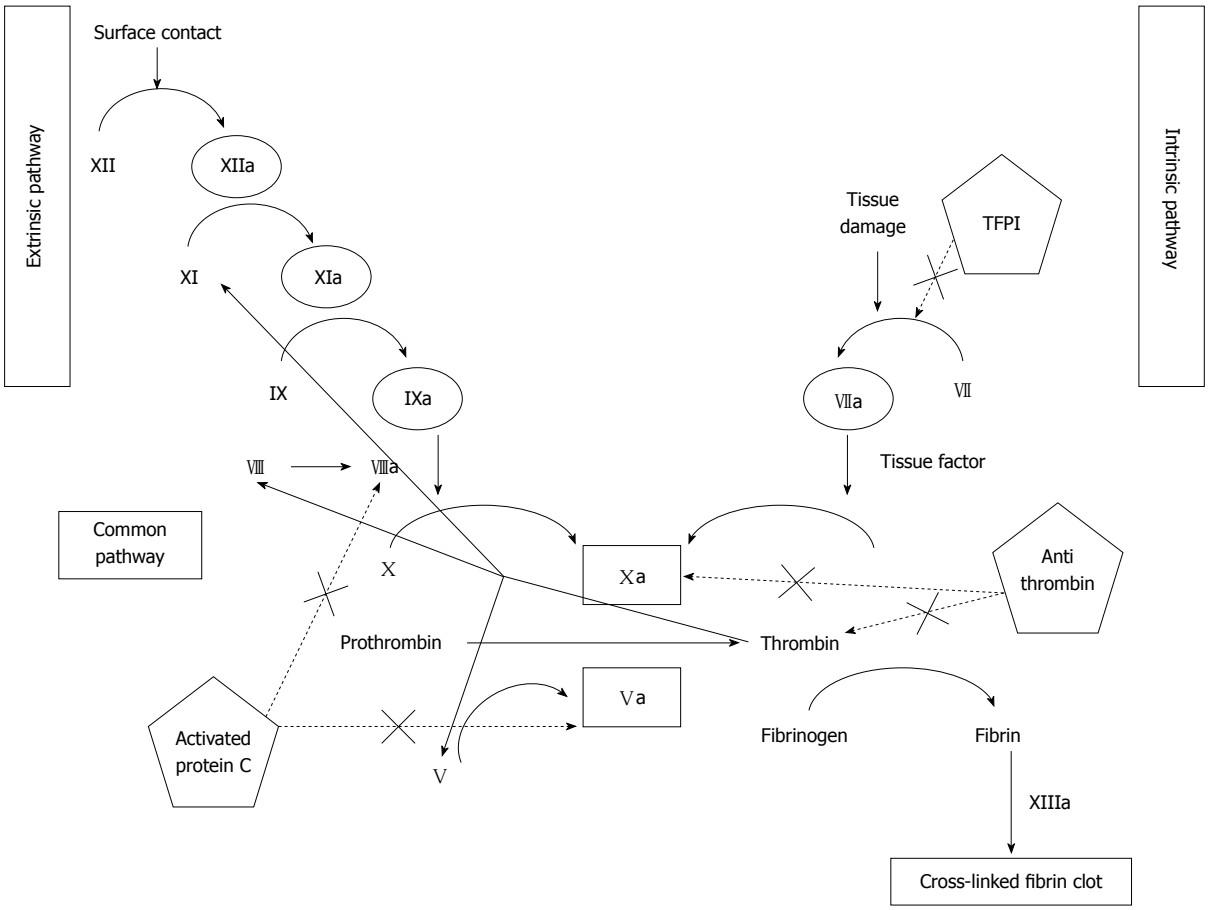

Figure 1 Coagulation cascade: Extrinsic, intrinsic, and common pathways. Coagulation factors are depicted in roman numerals with the suffix "a" denoting activated forms of the factors. Inhibitory pathways are shown in dotted lines. TFPI: Tissue factor pathway inhibitor.

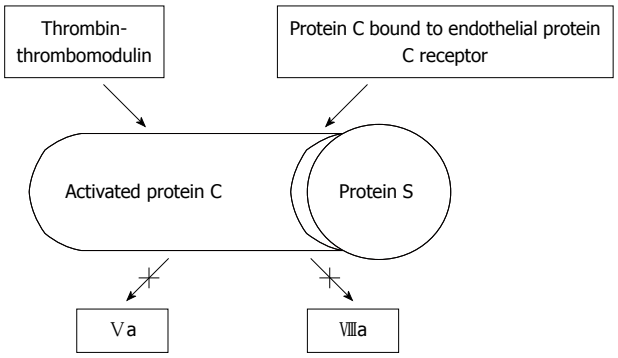

Figure 2 Anticoagulant effect of thrombomodulin, protein C, and protein S.

virus is able to activate hemostasis through several mechanisms, one of the main processes being HCV-induced endothelial damage and/or activation. Several clinical observations that are outlined below support this evidence (Figure 3).

In 1996, Prieto *et al*[8] reported an increased prevalence of anticardiolipin antibodies among HCV patients. This increased prevalence was related to prior thrombotic episodes, portal hypertension and thrombocytopenia. In a study on 201 patients, 124 of them with HCV infection, Biron *et al*[9] found a 33% prevalence of antiphospholipid antibodies that were significantly associated with increased liver fibrosis assessed by METAVIR fibrosis score. A relationship between HCV infection and prothrombotic state was also evidenced by Enger *et al*[10] (2014), who found that HCV infection was associated with various kinds of thromboembolic diseases in a cohort of 22733 HCV infected individuals in the United States. The incidence rate ratio among non-cirrhotic HCV infected patients was 1.44 (95%CI: 1.31-1.58) for any thrombotic event, and reached 6.06 (95%CI: 2.04-18.01) for portal vein thrombosis. Wang *et al*[11] compared 3686 HCV-infected patients with 14744 subjects without HCV or HBV infection and followed them during more than 5 years. They found that HCV infection was associated with a hazard ratio of deep vein thrombosis of 1.96 (95%CI: 1.03-3.73). Chen *et al*[12] in 2013 found a higher prevalence of pulmonary hypertension among HCV patients, portal vein thrombosis (a feature commonly present in their series of HCV patients) being an independent factor. However, other authors have failed to find a relationship between antiphospholipid antibodies and thrombotic episodes in HCV patients[13,14], although antiphospholipid antibodies were more frequently observed among HCV patients. Already in 1995, Violi *et al*[15] performed a study on 18 patients with liver cirrhosis and 36 controls and found that

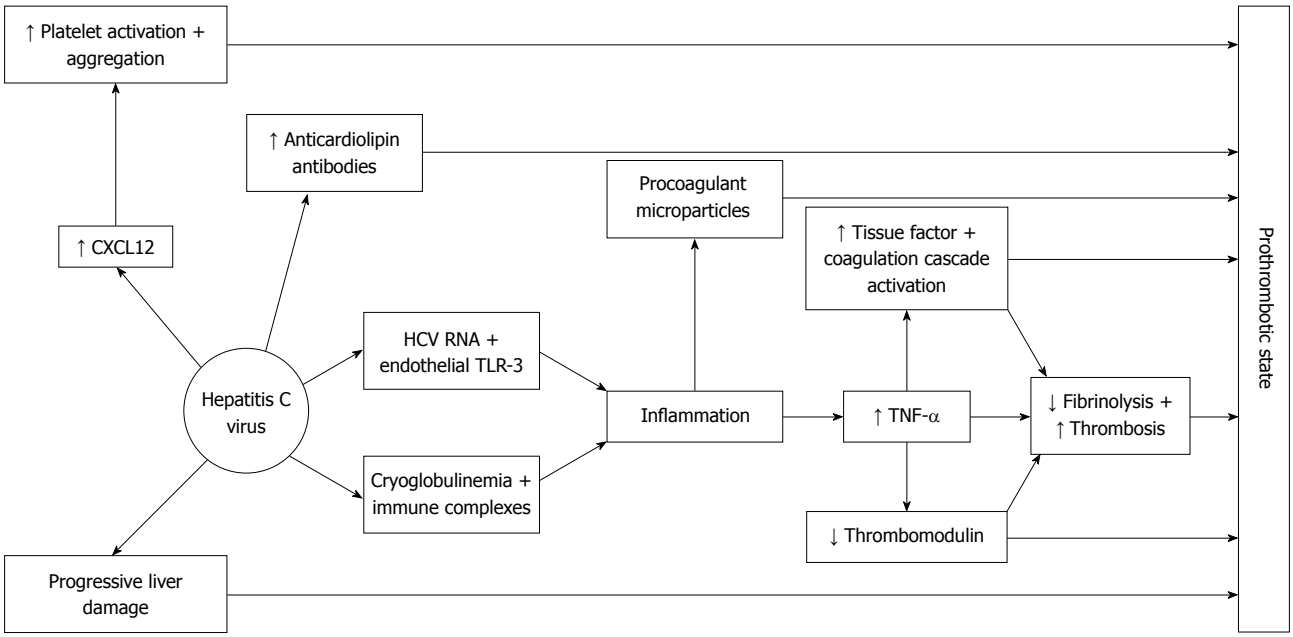

Figure 3 Hepatitis C virus has several effects on anticoagulant and procoagulant cascades, in addition to the effects on coagulation derived from liver cirrhosis development. (1) Hepatitis C virus (HCV) infection is associated with anticardiolipin antibodies which are related to thrombotic events; (2) Viral HCV RNA binds to toll-like receptors (TLR)-3 found in endothelial cells which leads to inflammation. Also, HCV infection is associated with cryoglobulinemia and thus immune complexes directed against viral RNA are formed. Inflammation generated by these two mechanisms lead to TNF-α secretion. TNF-α is an inducer of tissue factor expression, therefore exerting a prothrombotic effect by activating the coagulation cascade and also downregulates thrombomodulin expression; and (3) In HCV infection, CXCL12 is up-regulated in the endothelium of blood vessels formed in active inflammatory foci. CXCL12 is a potent promoter of platelet aggregation and adhesion.

venous thrombosis was associated with HCV infection, positive antiphospholipid antibodies, and increased rate of thrombin generation.

Main mechanisms involved

The mechanisms that may contribute to thrombosis in HCV infection include: systemic inflammation due to viral infection, direct infection of endothelial cells, viral-induced down regulation of physiological anticoagulant mechanisms, and alterations of fibrinolysis and thrombin generation dependent upon tissue factor generation by the infected endothelium[16]. The generation of antiphospholipid antibodies associated with HCV infection may also favor the generation of a procoagulant milieu[8], and immune complexes, usually in relation to cryoglobulinemia, may also trigger thrombotic phenomena[17].

Direct effect of HCV on anticoagulant and procoagulant pathways

Activation of tissue factor is of primary importance in the initiation of the coagulation cascade; and, as previously mentioned, damaged endothelium is the main source of tissue factor. Endothelial damage takes place in HCV infected patients, mainly by two main mechanisms. Firstly, today it is well known that HCV viral RNA binds to toll-like receptor (TLR)-3 in endothelial cells leading to inflammation[18] and generating enhanced expression of both tumor necrosis factor (TNF)-α and TNF receptor 2. In advanced stages of the disease, endothelial cells also express the chemokine CXCL 12 that recruits immune cells[19]. Additionally, endothelial damage associated with cryoglobulinemia is due to a type 3 hypersensitivity reaction with formation of immune complexes of antibodies directed against viral RNA. These immune complexes activate endothelial cells[20]. Inflammation generated by either of these mechanisms is accompanied by TNF-α secretion. TNF-α is an inducer of tissue factor expression, therefore exerting a prothrombotic effect[21] and also down-regulates thrombomodulin expression[22]. Cytokines decrease fibrinolytic properties of the endothelial cells[23]. In addition, tissue factor is present in increased amounts in microparticles in patients with chronic pure HCV infection[24], contributing in this way to enhanced coagulation.

HCV infection and platelets

Hemostatic platelet function is also enhanced. In patients affected by HCV infection, CXCL12 is up-regulated in the endothelium of blood vessels formed in active inflammatory foci. It is elevated in the plasma of patients with marked fibrosis and avidly binds to CXCR4 overexpressed by liver infiltrating lymphocytes[19]. CXCL12 is a potent promoter of platelet aggregation and adhesion[25], and, indeed, increased platelet activation and aggregation have been described among HCV patients[26,27].

Perhaps this increased platelet aggregation may explain some striking features described in HCV

patients regarding platelet count. Thrombocytopenia is more marked in HCV-infected patients than among those affected by other forms of chronic liver disease in a similar stage of severity[28]. The reasons are not well understood[29,30], and there are reports in which thrombocytopenia improves after treatment with interferon alpha[31]. This effect is seen despite the fact that thrombocytopenia is a well known side effect of interferon alpha, which strongly suggests a direct causal relationship between thrombocytopenia and HCV infection. Theoretically, thrombocytopenia could be due to decreased production, but results regarding thrombopoietin levels are disparate. Español *et al*[32], in 2000, found normal values in 23 HCV patients with chronic hepatitis compared with 43 controls, but there are also reports pointing to decreased thrombopoietin levels in relation with progression of liver disease[33,34], and restoration of megakariopoiesis after successful liver transplantation[35]. These data suggest that liver function is essential for the maintenance of normal thrombopoietin levels, although it has been also reported that thrombopoietin may be degraded in excess by the enlarged spleen[36]. Thrombocytopenia could also be related to hypersplenism, but although splenic sequestration surely plays a role in advanced stages of liver disease, thrombocytopenia is already evident before spleen enlargement ensues. Antiplatelet antibodies have been described in HCV infection[37], but their pathogenetic role in thrombocytopenia is debatable[38]. However, in some studies the severity of the disease is accompanied by a progressive decrease of platelet production and an increase in platelet antibodies. Platelet count was inversely related to liver fibrosis and directly to viral load[39].

In any case, increased coagulation and decreased fibrinolysis, together with enhanced platelet aggregation may underlie the aforementioned well described relationship between HCV infection and venous thrombosis. When liver damage evolves during the natural history of chronic HCV hepatitis, further mechanisms add to those described.

Prothrombotic alterations associated with liver cirrhosis

Complications of liver cirrhosis probably account for most of the hospital admissions of patients with advanced HCV liver disease. As other forms of liver cirrhosis, cirrhosis in patients affected by chronic HCV infection is characterized by progressive fibrous tissue deposition in the liver that separates groups of hepatocytes forming nodules with distorted vascular architecture and variable degrees of necrosis and liver cell regeneration[40].

Two syndromes converge in this disease: liver failure and portal hypertension, leading to a constellation of clinical and laboratory alterations, some of them theoretically associated with increased bleeding risk. Among these, the most outstanding features related to portal hypertension include the development of oesophageal varices, hypertensive gastritis, hemorrhoids, and hypersplenism; secondary thrombocytopenia is usually found in these patients. Liver failure impedes correct synthesis of clotting factors, such as prothrombin or, in later stages, fibrinogen. Therefore, both syndromes may cause bleeding diathesis. However, in stark contrast, thromboembolic events are not unusual in cirrhosis[41,42], with an overall incidence of 0.8% of non portal vein thrombosis in a study on 2074 cirrhotic patients[43]. In that study, although 5 out of the 17 affected patients showed antiphospholipid antibodies, none of them showed mutation of factor V and/or prothrombin, so no other classic prothrombotic abnormality was identified. On the other hand, the incidence of portal vein thrombosis is by far higher among cirrhotics than among the general population, reaching prevalence values of 0.6%-5%, increasing up to 40% among patients with advanced disease, or 10%-25% according to other reports[44]. Local factors, such as venous stasis and portal hypertension-related endothelial dysfunction are clearly involved in this type of venous thrombosis, but it is worth of note that considering all the forms of venous thromboembolic disease together, cirrhotic patients showed an increased risk, that was more marked among patients with cirrhosis categorized as Child class C, despite a more deranged prothrombin activity[45].

Therefore, liver cirrhosis, despite a usually observed low platelet count and deranged synthesis of some clotting factors, may be considered a prothrombotic condition.

Anticoagulant and procoagulant pathways in cirrhosis

Independent of etiology, recent research has pointed out that in liver cirrhosis several alterations predispose to an increased thrombotic risk, especially at the portal vein.

Synthesis of antithrombotic proteins, such as antithrombin, protein C and protein S is more intensely deranged than that of procoagulant proteins. As mentioned earlier, protein C plays a major role in controlling coagulation. In its active form it degrades several coagulation factors, especially factor V, leading to a decrease in thrombin production. Decreased synthesis of protein C by an impaired liver function may favor ongoing thrombin formation[46]. Binding of thrombomodulin to protein C increases the speed of protein C activation. Interestingly, in a previous study we found raised levels of thrombomodulin among cirrhotics, in the face of decreased protein C, protein S and antithrombin[47]. However, thrombomodulin-bound thrombin also shows a prothrombotic effect, since it inhibits fibrinolysis by cleaving TAFI into its active form[48]. Therefore, raised thrombomodulin, *via* its action on TAFI, can be viewed as another factor potentially involved in the procoagulant milieu of liver cirrhosis.

Thrombin activation may be aggravated in some

situations in which anticoagulant pathways are further impaired. Factor Ⅴ Leiden is a common (2%-15% prevalence among Caucasians) autosomal dominant trait[49]. It carries a single mutation at position 506 that makes it resistant to the degradative action of activated protein C. As a consequence, the action of factor Ⅴa on thrombin synthesis increases, leading to a procoagulant state. Indeed, factor Ⅴ Leiden is associated with an increased risk of portal vein thrombosis both in patients with and without cirrhosis[50]-although there are studies that do not support this finding[51]. In addition, in patients with HCV infection who also bear factor Ⅴ Leiden polymorphism there is an increased rate of liver fibrous tissue deposition[52], whose underlying mechanisms will be discussed later. Poujol-Robert *et al*[53], in 2004, reported an increased odds ratio for cirrhosis among patients with HCV infection and factor V Leiden mutation, and Papatheodoridis *et al*[54] (2003) found that the presence of activated protein C resistance was associated with more intense fibrosis in patients with chronic viral hepatitis. Moreover, factor Ⅴ Leiden also carries an increased risk of fibrosis in other tissues, as shown by Xu *et al*[55] (2001) in pulmonary fibrosis that developed in bleomycin-treated mice carrying the factor Ⅴ Leiden mutation: both homozygous and heterozygous animals showed a nearly 40% increase in hydroxyproline excretion compared to wild-type mice.

Other factors may contribute to this pro-coagulant effect. Persistent or chronic inflammation is a thrombophilic condition, characterized by raised fibrinogen and factor Ⅷ, which are main contributors to this procoagulant milieu. Cirrhotics show raised levels of factor Ⅷ[56]. Also, cirrhotics have raised von Willebrand factor, which may favor a greater platelet adhesion[57]. Lipoprotein receptor-related protein is responsible for catabolism of factor Ⅷ. Its expression is decreased in cirrhotics[58]. In a similar fashion, ADAMTS-13, a metalloprotease involved in the catabolism of von Willebrand factor, is reduced in patients with liver cirrhosis[59]. Increased fibrinolysis related to decreased PAI-1 levels in relation to t-PA were also reported in cirrhotics[60], and a parallel deficiency in other mediators, such as TAFI, probably contributes[61]. It is currently accepted that hyperfibrinolysis may affect 30%-50% of cirrhotics with advanced disease[62].

Endothelial alterations of the portal vein radicles are well described in liver cirrhosis[63]. Endotoxaemia possibly plays a relevant role in endothelial alterations[64], independent on the eventual direct effects of HCV infection. As mentioned above, altered endothelium promotes coagulation by activation of tissue factor. In cirrhotics there is also an increase in the expression of several adhesion molecules, including platelet-endothelial cell adhesion molecule-1 (PECAM-1), L-selectin and P-selectin[65], and, as just mentioned, increased levels of von Willebrand factor[57].

Activated endothelial cells, as well as monocytes and platelets, also lead to the formation of microparticles that also carry tissue factor. In addition, platelet derived microparticles are able to transfer the GⅡb-Ⅲa platelet receptor to leukocytes, a feature which leads to the activation of the nuclear transcription factor kappa B, inducing gene transcription of proinflammatory mediators[66]. In addition platelet microparticles are able to carry factor Ⅴ[67]. Some studies point to an increased production of microparticles derived from leukocytes, lymphocytes, erythrocytes or even hepatocytes in liver cirrhosis[68]; despite some assertions[69], other researchers have failed to find raised platelet-derived microparticles in cirrhotic patients[70].

In summary, cirrhotics show more depressed levels of anticoagulants than those of procoagulants; although the role of microparticles in liver cirrhosis is unclear, portal hypertension-related endothelial damage and endotoxin-mediated cytokine activation, together with altered fibrinolysis in some cases, all contribute to the prothrombotic state of these patients, with increased thrombin formation.

Platelets are also altered in liver cirrhosis, both in number and function. Decreased platelet number may be due to splenic pooling due to portal hypertension, lower thrombopoietin levels, and increased consumption due to endotoxaemia, increased antiplatelet antibody production, and increased coagulation activation[69], but no significant bleeding takes place if platelet count is over 60000/fl[71]. Qualitative platelet alterations include defective adhesion[72], partially compensated by increased von Willebrand factor; decreased aggregation in response to normal stimuli, such as ADP, ristocetin, thrombin, collagen or epinephrine[73] and altered function[26] which progresses as liver function worsens. The aforementioned increased production of prothrombotic platelet-derived microparticles may compensate for these defects, so that hemostasis does not become significantly altered. On the contrary, portal thrombotic events may occur if a thrombopoietin receptor agonist (Eltrombopag) is administered[74], despite a median maximum platelet count of 148000/fl.

All these factors add to the previously mentioned direct effects of HCV infection, explaining why in HCV liver cirrhosis the thrombotic complications are more frequently observed than in cirrhosis of other etiologies.

CONSEQUENCES OF INCREASED THROMBIN FORMATION

Thrombin and fibrous tissue deposition: Direct effects on fibrogenesis

Thrombin not only plays a central role in the coagulation system. The importance of increased thrombin activation in patients with liver cirrhosis resides in the fact that thrombin may be directly involved in fibrogenesis (Figure 4). Thrombin exerts this action after binding to a group of receptors called protease activator receptors (PAR). There are four of such

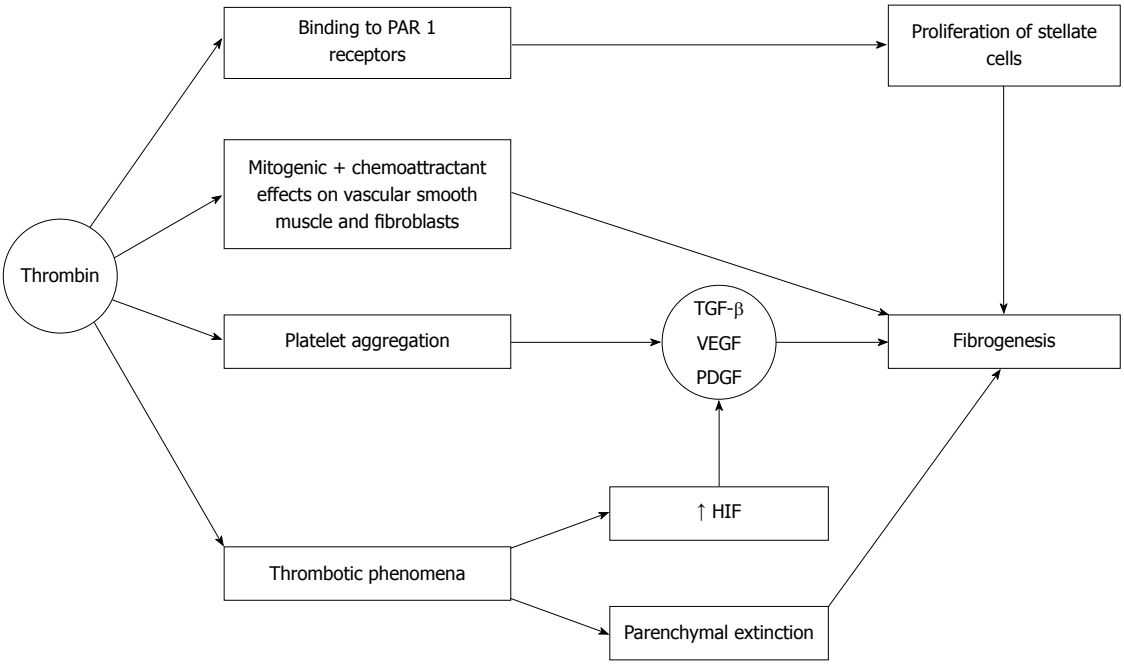

Figure 4 Effects on thrombin on fibrogenesis. Thrombin binds to PAR-1 receptors on hepatic stellate cells which leads to proliferaton and activation of these cells Thrombin also promotes platelet aggregation. Platelet alpha granules are rich in several growth factors, including TGF-β, which in turn promotes fibrogenesis. Vascular endothelial growth factor (VEGF) and PDGF also play contributory roles. Thrombin also exerts mitogenic and chemoattractant effects on vascular smooth muscle cells and fibroblasts. Finally, thrombotic phenomena also occur within the liver, leading to ischemic parenchymal injury, and substitution of parenchyma by fibrous tissue (the so called parenchymal extinction). When a clot provokes ischemia, VEGF, PDGF and TGF-β are activated probably *via* an increase in hypoxia-inducible-factor (HIF), which is raised in cirrhosis in relation to portal microthrombotic phenomena.

type of receptors (1-4), which become activated by several different proteases including thrombin (which activates PAR 1, 3, 4), and tryptase, which activates PAR-2, providing a rational basis to sustain the finding of liver fibrosis in the context of systemic mastocytosis[75]. PAR-1 receptors are present in the liver, and increase their expression in advanced liver disease[76] and along the transformation of stellate cells into myofibroblasts[77]. Indeed, hepatic stellate cells are one of the main cells in which these receptors become up-regulated in chronic liver disease. Binding of PAR with their ligands on stellate cells leads to proliferation and activation of these cells, that secrete monocyte chemoattractant protein 1 (MCP-1), increase deposition of extracellular matrix and the expression of receptors for platelet derived growth factor (PDGF) and transforming growth factor (TGF)-β[78]. In addition MCP-1 attracts monocytes, that produce tissue factor which contributes to more thrombin generation[79].

Another well known effect of thrombin is the ability to promote platelet aggregation. Platelet alpha granules are rich in several growth factors, including TGF-β, which in turn promotes fibrogenesis. Vascular endothelial growth factor (VEGF) and PDGF also play contributory roles. PDGF is a very strong mitogen for stellate cells and it also promotes fibrogenic activity by these cells[80]. PDGF upregulates the expression of matrix metalloproteinase (MMP)-2, MMP-9 and tissue inhibitor of metalloproteinase (TIMP)-1 and downregulates that of collagenase[40].

Thrombin also exerts mitogenic and chemoattractant effects on vascular smooth muscle cells and fibroblasts[81]. Factor Xa also activates PAR-1 and PAR-2, and may lead to fibrous tissue deposition. It was shown that incubation of fibroblasts with factor Xa led to a 12.6-fold increase in TGF-β expression, an increase which was by far more intense than that elicited when the cells were stimulated by thrombin[82].

Therefore, thrombin, both directly and indirectly, together with other activated coagulation factors, may play a role in the progression of liver cirrhosis, *via* the described effect on fibrogenesis.

Thrombin and clot formation: Parenchymal extinction and fibrogenesis

In addition to thrombosis of major veins, microthrombotic phenomena also occur within the liver, leading to ischemic parenchymal injury, and substitution of parenchyma by fibrous tissue (the so called parenchymal extinction[83]). This phenomenon consists in the ischemic collapse of hepatocytes between portal vein radicles and hepatic central venule, and becomes strongly exacerbated if congestive phenomena coexist, such as heart failure[84]. Coalescence of neighbouring tracts could lead to the formation of gross fibrous tracts and the evolution to cirrhosis[85]. When a clot provokes ischemia, VEGF, PDGF and TGF-β are activated[86] probably *via* an increase in hypoxia-inducible-factor (HIF), which is raised in cirrhosis due to increased portal resistance[87].

However, there are studies that do not support a pathogenetic role of portal vein thrombosis on progression of liver disease[88], but, as discussed above, factor V Leiden seems to accelerate progression of fibrosis in HCV infected patients. In other studies, other prothrombotic conditions such as hyperhomocysteinaemia[89] or mutations in factor XIII (both as an isolated finding or in combination with PAI-1 4G/5G mutation) also constitute a risk factor for an increased rate of liver fibrosis development in patients affected with HCV or chronic hepatitis B[90]. In other studies, the mutation associated with increased fibrosis progression rate was the prothrombin G20210 A mutation[91].

These data support the importance of microthrombotic phenomena in the progression of liver disease, especially in HCV-infected patients, in whom the endothelial changes promoted by HCV may be considered triggering factors, aggravated in later stages of the disease by the endothelial changes secondary to portal hypertension.

Therapeutic future prospects

Based on the aforementioned data, low molecular weight heparin has been advocated as a therapeutic option in patients with cirrhosis, with promising results: enoxaparin for 48 wk not only significantly prevented the development of portal vein thrombosis in patients with cirrhosis with Child-Pugh scores between 7 and 10, but it also associated with a decreased probability of decompensation of liver disease[92]. In that study, 18 out of 34 randomized to enoxaparin and 18 out of 36 in the placebo group were HCV-infected patients. Experimental research in rats has shown that treatment with heparin reduced severity of biochemical and histological changes induced in rats with oral carbon tetrachloride[93]. The possible role of anticoagulation in the treatment of advanced liver disease, especially in HCV-induced cirrhosis, is a promising idea that warrants confirmation[94].

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TOPIC HIGHLIGHT

2016 Liver Transplantation: Global view

Liver transplantation: Current status and challenges

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Abstract

Great progress has been made in the field of liver transplantation over the past two decades. This progress, however, also brings up the next set of challenges: First, organ shortage remains a major limitation, and accounts for a large proportion of wait list mortality. While living donation has successfully increased the total number of liver transplants done in Asian countries, the total number of such transplants has been stagnant in the western hemisphere. As such, there has been a significant effort over the past decade to increase the existing deceased donor pool. This effort has resulted in a greater use of liver allografts following donation after cardiac death (DCD) along with marginal and extended criteria donors. Improved understanding of the pathophysiology of liver allografts procured after circulatory arrest has not only resulted in better selection and management of DCD donors, but has also helped in the development of mechanical perfusion strategies. Early outcomes demonstrating the clinical applicability of both hypothermic and normothermic perfusion and its potential to impact patient survival and allograft function have generated much interest. Second, long-term outcomes of liver transplant recipients have not improved significantly, as recipients continue to succumb to complications of long-term immunosuppression, such as infection, malignancy and renal failure. Furthermore, recent evidence suggests that chronic immune-mediated injury to the liver may also impact graft function.

Key words: Donation after cardiac death; Mechanical perfusion; Renal-sparing immunosuppression; Antibody-mediated rejection

Core tip: Organ shortage remains a major limitation in liver transplantation, and there has been a significant effort over the past decade to increase the existing deceased donor pool. Recent advances have included better selection and management

of donors after circulatory arrest, application of hypothermic and normothermic perfusion, minimization of standard immunosuppression and use of new immunosuppressive medications. Additionally, there has been renewed emphasis and understanding of liver immunology and the impact of antibody-mediated rejection. Together, these advances have allowed for expansion of the donor pool with concurrent improved patient outcomes.

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INTRODUCTION

Over the last several decades, the field of transplantation has witnessed much change yet, among all solid organ transplants, arguably the greatest advances have been in liver transplantation. Today, liver transplantation is universally accepted as the only treatment option for end-stage liver disease, acute fulminant hepatic failure, hepatocellular carcinoma, hilar cholangiocarcinoma and several metabolic disorders. Advancements in surgical technique, perioperative management and immunosuppressive therapy have yielded excellent short-term graft and patient survival outcomes which have become the expected norm. With these advances comes the next set of challenges: organ shortage and improving long-term outcomes of the liver transplant recipients.

ORGAN SHORTAGE

The disparity between the number of available liver allografts and transplant candidates continue to grow worldwide. In Asia, this problem has been successfully addressed by ever-increasing numbers of living-donor liver transplantation (LDLT). In the western countries, however, the number of LDLT has not seen a significant change for over a decade, and the demand for deceased donor liver allografts continue to increase. Thus, substantial effort has been put in to expand the existing deceased donor pool.

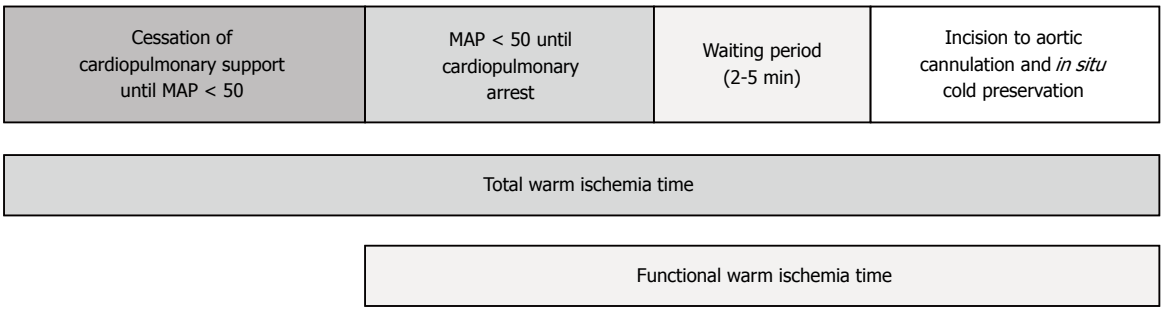
Use of donation after cardiac death liver allografts

In the early 1990s, the use of livers procured from donation after cardiac death (DCD) donors was an early attempt to narrow the disparity between organs and recipients in need[1]. The early experience with DCD livers was not favorable, as prolonged donor warm ischemia time and ischemia-reperfusion injury likely played a large role in the high rate of primary non-function, hepatic artery thrombosis, ischemic cholangiopathy, and allograft failure[2,3]. With increasing experience, however, factors associated with improved outcomes have been identified and more centers have begun to use these donors[4-6].

To date, there have been several large retrospective analyses which have identified DCD donor-related variables associated with recipient outcomes (Figure 1). Young age (45 ± 10 years old), short donor warm ischemia time (less than thirty minutes), and limited cold ischemia time (less than ten hours) have been shown to improve allograft outcomes[4-6]. In the Mayo Clinic experience, every minute added to the warm ischemia period between asystole and cross-clamp was found to be associated with a 16.1 percent increase in the odds of ischemic cholangiopathy[6]. Recipient factors associated with improved DCD outcomes generally concentrate on avoidance of sicker patients: excluding patients requiring retransplantation, patients with renal failure (creatinine > 2.0 mg/dL) and patients on life support[5]. The correlation between these factors and outcomes is likely related to minimization of both cold ischemia time and further allograft injury through recipient instability. As a result, by applying these donor and recipient criteria, DCD outcomes have improved considerably with recipient survival and allograft function similar to that of donation after brain death (DBD) donors[4-6].

Mechanical perfusion of deceased donor liver allografts

Despite these improved outcomes, many DCD livers continue to go unused as a result of unacceptable donor parameters and concern for poor allograft function. The concept of mechanical perfusion for solid organ transplantation was originally introduced in the late 1960s by Belzer *et al*[7] and renewed interest returned following a report by Moers *et al*[8] in 2000 which, through a prospective randomized control trial, demonstrated a decreased incidence of delayed graft function and improved graft survival in in kidney transplant recipients. Since then, hypothermic machine perfusion in kidney transplantation has gained a widespread use. Although suboptimal, static hypothermic cold storage remains the primary method for liver preservation, largely because of its cost effectiveness, simplicity, and logistics. At present, there is a large and apparent need to optimize preservation particularly for DCD, marginal, and extended-criteria donor organs. These allografts are subject to a greater risk of ischemia-reperfusion injury which occurs as a result of donor warm ischemia time, aortic cross-clamping and initiation of cold ischemia, rewarming during graft implantation, and finally full reperfusion (Figure 1). It is here that the utility of mechanical perfusion has emerged as potential solution to this problem.



Ref.	Txp Period	No. Patients	Donor Age	WIT	CIT	Asystole-to-cross clamp
4	1996-2006	874	⩽ 45 yr	⩽ 15 min[1]	⩽ 10 h	
5	1996-2003	367	-	< 30 min[1]	< 10 h	
6	1998-2010	200	-	-	-	Minimization of asystole-to-cross clamp time (1 min ≈ 16.1% IC)

Figure 1 Favorable donation after cardiac death donor criteria. [1]From time of cessation of cardiopulmonary support to with loss of hemodynamic and respiratory function up to the *in situ* initiation of cold preservation solution. MAP: Mean arterial pressure; WIT: Warm ischemia time; IC: Ischemia cholangiopathy.

Table 1 Comparison of hypothermic and normothermic mechanical perfusion

	Hypothermic mechanical perfusion	Normothermic mechanical perfusion
Temperature	4 ℃	37 ℃
Pathophysiology	Reduced cellular metabolism Non-functioning liver during preservation	Continued cellular metabolism Functioning liver during preservation
Perfusate	No specific requirements, oxygen can be used	Continuous delivery of nutrients and oxygen needs to be maintained
Logistics	Similar to that of hypothermic mechanical perfusion currently used for renal allografts	More complex
Benefits	Logistically easier May provide benefit in marginal livers	Allows for the assessment of metabolic and synthetic function during preservation May provide benefit in marginal livers Potential use in steatotic livers Felt to reduce ischemia reperfusion injury

Hypothermic perfusion

Hypothermia slows cellular metabolism and prolongs the amount of time an organ can be deprived of oxygen without loss of viability (Table 1). In liver transplantation, hypothermic perfusion has shown similar benefits. Authors Guarrera *et al*[9] have demonstrated that hypothermic perfusion decreases the extent of graft injury and subsequent clinical studies by the same group have shown improved allograft function, lower serum transaminases and decreased hospital stay as compared to matched historic cold storage liver allografts from DBD donors[10,11]. Equally beneficial results have been reported in DCD liver allografts[12,13]. In a trial using human DCD livers, Hypothermic Oxygenated PErfusion (HOPE) was applied for 1 to 2 h prior to implantation[12]. Functional warm ischemia time (MAP < 50 mmHg to cold flush) in this group ranged from 22 to 41 min and postoperative allograft function was normal in the entire cohort. In a follow-up period of 8.5 mo, no evidence of intrahepatic biliary complications was noted. As a continuation to this study, authors Dutkowski *et al*[13] recently published their results evaluating DCD livers treated with HOPE along with matched static cold storage DCD livers. As anticipated, results for DCD livers subjected to HOPE were superior with decreased graft injury, decreased intrahepatic cholangiopathy and biliary complications and improved 1-year graft survival[13]. Similarly, hypothermic machine perfusion has also been applied to extended criteria DBD donor liver allografts with encouraging outcomes, such as decreased allograft dysfunction (19% *vs* 30%), improved patient survival at one year (84% *vs* 80%) and a reduction in biliary complications (13% *vs* 43%)[11].

Normothermic perfusion

Normothermic perfusion is technically more demanding, as it requires a continuous delivery of nutrients and oxygen in order to maintain ongoing cellular metabolism[14]. In theory, this setup reduces the ischemia-reperfusion injury and may thereby allow for the safe transplantation of marginal liver allografts. Normothermic mechanical perfusion can also allow for the assessment of liver function during the pre-

implantation period (Table 1). Although these advantages are notable, historically, there have been difficulties in maintaining stable perfusion[15]. In a preclinical work by op den Dries *et al*[16], discarded DCD human donor livers were subjected to normothermic mechanical perfusion following cold ischemia times of 6.9 ± 1.9 h (WIT 15.5 ± 2.4 min). After six hours of normothermic perfusion (37 ℃), bile production was observed and histological examination showed well-preserved liver morphology without signs of hepatocellular ischemia, biliary injury, or sinusoidal damage[16]. Currently, there are several human trials underway comparing normothermic liver preservation with static cold storage. The first Phase I, non-randomized, prospective trial using the normothermic mechanical perfusion unit enrolled twenty transplanted livers in United Kingdom and was completed in 2014. Results from this trial demonstrated the safety and feasibility of normothermic machine preservation in human liver allografts (personal communication). In the interim, a multicenter, randomized controlled trial with enrollment plan for 260 liver allografts (130 using OrganOx©; 130 using static cold storage) is under way.

Steatotic liver allografts and mechanical perfusion

The application of mechanical perfusion to marginal donors is foreseeable as hepatic steatosis has become increasingly common in the United States and the incidence of deceased donors with steatotic livers has concurrently increased. Allografts with severe steatosis, defined as greater than sixty percent of the parenchyma, are known to carry a high risk of primary non-function[17,18]. On the contrary, liver allografts with mild steatosis (< 30%) yield results similar to those of non-steatotic liver allografts[17,18]. The outcomes of liver allografts with moderate steatosis (30% to 60%) remain variable, and often depend upon additional factors such as recipient stability, ischemia time, and mechanism of donation (*i.e.*, DBD *vs* DCD). Impaired microcirculation secondary to increased hepatocyte volume is theorized to be responsible for the relative susceptibility of steatotic livers to ischemia[19]. Cellular edema accompanying ischemia-reperfusion likely results in further obstruction of the sinusoids, thereby exacerbating this injury. Accordingly, steatotic DCD liver allografts are generally discarded as these scenarios combine several risk factors. As such, the application of mechanical perfusion may help salvage steatotic livers. At present, preclinical and clinical studies are lacking however Bessems *et al*[20] evaluated mechanical perfusion using a steatotic rat model in which they compared static cold storage and hypothermic oxygenated mechanical perfusion. Results from this animal model found that preservation of steatotic livers stored in standard cold storage resulted in more cellular injury. By comparison, the steatotic livers subjected to hypothermic oxygenated mechanical perfusion showed improved bile production and higher ATP levels[20].

IMPROVING LONG-TERM LIVER TRANSPLANTATION OUTCOMES

Although the short-term outcomes after liver transplantation have been excellent worldwide, long-term outcomes remain suboptimal. The top causes of late mortality after liver transplantation are allograft failure, cardiovascular events, infection, malignancy and renal failure[21]. There is a clear and direct link between these and the long-term use of immunosuppressive medications. As a result, in the past decade, a significant effort has been put forth to minimize immunosuppression in liver transplantation.

Renal sparing protocols

Renal insufficiency is the strongest predictor of late mortality following liver transplantation[21]. Because of this, the use of renal sparing immunosuppression protocols both during the induction and maintenance periods following liver transplantation has been introduced into the clinical practice over the past decade.

Mammalian target of rapamycin inhibitors: A number of large trials evaluated the role of mammalian target of rapamycin inhibitors (mTOR) in liver transplantation. The Preservation of Renal Function in Liver Transplant Recipients with Certican Therapy (PROTECT) study[22], a randomized controlled trial sought to evaluate the effect of conversion from calcineurin inhibitors (CNI) to everolimus starting four weeks following liver transplantation. Unfortunately, at one year, the study results proved to be inconclusive. No difference in glomerular filtration rater (GFR) was noted using the Cockcroft-Gault formula. Moreover, despite lack of clear benefit, the study did, however, observe a higher rate of infections, anemia, leukopenia and hyperlipidemia in the Everolimus treatment group[22].

By comparison, a second trial by Masetti *et al*[23] in 2010 showed some benefit in using mTOR inhibitors in liver transplantation. In this randomized control trial, cyclosporine (CsA) was used for ten days following liver transplantation. Patients were then randomized to Everolimus or cyclosporine plus mycophenolate mofetil (MMF). At twelve months, GFR was better in the Everolimus group and no differences were noted in patient survival, acute rejection or hepatic artery thrombosis[23]. Among the patients with a GFR less than 60 mL/min per 1.73 m^2, the Everolimus treatment group showed a benefit. Accordingly, conclusions of the study were that early withdrawal of CsA followed by Everolimus monotherapy in de novo liver transplant patients was associated with an improvement in renal function, although, a criticism of the study has been the baseline difference between

the two groups with regard to the starting GFR.

In Spare-the-Nephron Trial[24], patients on CNI/MMF for the first four weeks post-transplant were randomized to either continue the same regimen or convert to Sirolimus/MMF. A mean increase in patient GFR was noted in the Sirolimus group, however the composite endpoint demonstrated non-inferiority of Sirolimus to CNIs when used with MMF[24]. Review of other smaller studies evaluating the conversion from CNI to mTOR inhibitors in liver transplant patients with renal insufficiency reveals similar variable results[25-28]. A meta-analysis of the use of Sirolimus in liver transplant recipients with CNI-induced renal insufficiency found no significant improvement in GFR, risk of death or graft failure[29]. Findings were, however, significant for an increased risk of infection, mouth ulcers and treatment discontinuation[29]. Given this variability, the role of mTOR inhibitors continues to evolve with much indecision and hesitancy surrounding their use for renal insufficiency. Newer roles, specifically with regard to benefit in hepatocellular carcinoma, likewise remain to be fully realized.

Induction therapy and delayed introduction of calcineurin inhibitors: Delayed introduction of CNI following liver transplantation may, theoretically, help decrease the negative impact of CNI on renal function[30-33]. Among *de novo* liver transplant recipients who had pre-existing renal insufficiency, Thymoglobulin induction and delayed introduction of CNI resulted in lower serum creatinine, a higher estimated GFR, and less dependence on dialysis at twelve months[30]. Similar strategies have been employed using newer generation anti-interleukin-2-receptor antibodies (Basiliximab, Daclizumab) for induction[32,33]. The ReSpECT trial, comparing standard Tacrolimus *vs* reduced-dose Tacrolimus and Daclizumab induction and delayed reduced-dose Tacrolimus[34], demonstrated that the greatest decline in eGFR was seen in the standard Tacrolimus group with the least decline noted in the Daclizumab induction and delayed reduced-dose Tacrolimus group. Currently, at Mayo Clinic, Basiliximab induction is used in patients with renal insufficiency so as to delay initiation of CNI post-transplant.

Immunosuppression minimization/withdrawal: Of all the solid organs that are transplanted routinely, liver allograft appears to be unique, as approximately 19 percent of the recipients can achieve "operational" tolerance (off immunosuppression incidentally or obligatorily)[35,36]. Given these findings, several recent trials investigated elective withdrawal of immunosuppression (IS) in liver transplant recipients. In one such trial, 12 out of 20 (60%) pediatric liver transplant recipients were successfully weaned off IS, with no or minimal portal inflammation[37]. In a large European multi-center trial of 102 adult deceased donor liver transplant recipients, 41 (40%) achieved operational tolerance[38]. At present, more information is needed so as to better guide clinical decision making and similar trials with long-term follow-up will help identify parameters with predictive and diagnostic value regarding who could be successfully weaned off IS. Longitudinal follow-up of operationally tolerant patients will also further demonstrate whether long-term outcomes improve with limiting the recipients' cumulative exposure to IS, particularly CNI.

Chronic antibody-mediated injury

Historically, liver allografts were thought to be spared from the HLA antibody-mediated injury, however recent findings have challenged this concept. The Baylor group initially reported their observation in liver recipients with chronic rejection, who had circulating donor-specific HLA-antibody (DSA) more often than in patients with no rejection[39]. Subsequently, they found that multiple IgG subclasses were found in the sera of chronic rejection patients, and the IgG3 subclass was associated with increased risk of graft loss[40]. A definitive cause-and-effect relationship remains to be demonstrated however, as these studies used post-transplant DSA for analyses and did not include protocol biopsies. In our own observation at Mayo Clinic, we did not find any DSA-mediated graft injury in the first year after transplantation[41], and over the long term, de novo DSA formation was preceded by liver allograft dysfunction (*e.g.*, recurrent HCV cirrhosis)[42].

Based on these new observations, it appears that DSA may indeed cause chronic liver allograft injury, although less frequently than in kidney or hear transplantation. In order to better understand the impact of DSA on both early and late outcomes in liver transplantation, longitudinal prospective studies, similar to those done in kidney transplantation, correlating DSA (both pre- and posttransplant at routine intervals) with clinical outcomes and graft histology through protocol biopsies will need to be done. These studies will ideally be designed to analyze DSA in detail, target antigen expression in the allograft, and take into account immunosuppression and compliance issues in the recipients. In addition, histologic and genetic evidence of endothelial cell injury and microvascular inflammation (hallmark of DSA-mediated injury in kidney allografts) will need to be investigated in liver transplant recipients with circulating DSA (Table 2)[43-58].

CONCLUSION

Along with the advances made in liver transplantation over the past thirty years have come a new set of challenges. As the demand for liver transplantation continues to grow worldwide, more collaborative studies are needed to narrow the disparity between the number of available deceased donor liver allografts and wait-listed patients, and to improve the long-term

Table 2 Current data for donor-specific HLA-antibody-associated injuries in kidney and liver transplantation

	Kidney transplantation	Liver transplantation
DSA specificity and levels	↑ risk hyperacute rejection[44]; ↑ DSA in new onset late kidney allograft dysfunction[45]; acute AMR is associated with high posttransplant DSA levels[45]	↑ DSA in recipients with CR, presence of IgG3 subclass associated with ↑ risk of graft loss[40]
C4d deposition in microvasculature	Graft failure significantly worse in the presence of C4d+ staining[44]; C4d+ is a marker of antibody-mediated injury[44,46]	C4d+ staining nonspecific; In the presence of DSA, linear portal capillary and sinusoidal staining observed in ACR; DSA negative C4d+ staining found in biliary strictures and recurrent liver disease[47]
DSA subtypes, C1q binding	↓ graft survival and ↑ risk for AMR with C1q-binding DSA[48,49]	Limited data[50]
Microvascular inflammation	Peritubular capillaritis is a possible predictor of chronic AMR[51]; subclinical AMR may contribute to development of CAN[52]	No current data
EC activation by light microscopy	EC and BM ultrastructural abnormalities in glomerular and peritubular capillaries are early markers of TXG[53]	No current data
Gene expression profile of chronic AMR	Defined genetic profile in AMR[54,55]	No current data
Therapeutic-trials to prevent DSA-associated injury	Bortezomib (plasma cell-targeted therapy) as a possible antihumoral therapy[56]; plasma exchange for AMR[57,58]	No current data

Modified from Ref. 43. AMR: Antibody-mediated rejection; CAN: Chronic allograft nephropathy; EC: Endothelial cell; BM: Basement membrane; DSA: Donor-specific HLA-antibody; TXG: Transplant glomerulonephropathy.

outcomes of the liver recipients.

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TOPIC HIGHLIGHT

2016 Pancreatic Cancer: Global view

Advances in inducing adaptive immunity using cell-based cancer vaccines: Clinical applications in pancreatic cancer

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Abstract

The incidence of pancreatic ductal adenocarcinoma (PDA) is on the rise, and the prognosis is extremely poor because PDA is highly aggressive and notoriously difficult to treat. Although gemcitabine- or 5-fluorouracil-based chemotherapy is typically offered as a standard of care, most patients do not survive longer than 1 year. Therefore, the development of alternative therapeutic approaches for patients with PDA is imperative. As PDA cells express numerous tumor-associated antigens that are suitable vaccine targets, one promising treatment approach is cancer vaccines. During the last few decades, cell-based cancer vaccines have offered encouraging results in preclinical studies. Cell-based cancer vaccines are mainly generated by presenting whole tumor cells or dendritic cells to cells of the immune system. In particular, several clinical trials have explored cell-based cancer vaccines as a promising therapeutic approach for patients with PDA. Moreover, chemotherapy and cancer vaccines can synergize to result in increased efficacies in patients with PDA. In this review, we will discuss both the effect of cell-based cancer vaccines and advances in terms of future strategies of cancer vaccines for the treatment of PDA patients.

Key words: Pancreatic cancer; Dendritic cell; Whole tumor cell; Cancer vaccine; Cytotoxic T lymphocyte

Core tip: Chemotherapy and cell-based cancer vaccines such as dendritic cell- and whole tumor cell-based cancer vaccines can synergize to result in increased efficacies in patients with pancreatic ductal adenocarcinoma (PDA). Moreover, cell-based cancer vaccines and immune checkpoint inhibitors can be used to block inhibitory ligand/receptor interactions by acting on certain cancer cells or T cells, allowing an enhancement of the antitumor immune response in specific tumors, including PDA. Therefore, the blockade of immune regulatory checkpoints combined with cell-based cancer vaccines and/or chemotherapy may be effective in inducing adaptive antitumor immunity in patients with PDA.

Kajihara M, Takakura K, Kanai T, Ito Z, Matsumoto Y, Shimodaira S, Okamoto M, Ohkusa T, Koido S. Advances in inducing adaptive immunity using cell-based cancer vaccines: Clinical applications in pancreatic cancer. *World J Gastroenterol* 2016; 22(18): 4446-4458 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i18/4446.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i18.4446

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDA), which is derived from glandular tissue of the pancreas, accounts for approximately 95% of pancreatic cancer and is one of the most lethal cancers because of a propensity for metastatic spread[1,2]. Although the definitive treatment for early-stage PDA is surgical resection, this is only possible in approximately 15% of cases[3], as most patients with PDA present in an advanced stage at the time of diagnosis. Additionally, despite surgical resection, radiation and/or chemotherapy, patients with PDA have an overall 5-year survival of only 5% due to local recurrence and metastasis[1,2,4]. PDA cells grow rapidly and spread outside of the pancreas, including into the liver, lung, bone, and brain, through lymphatic and/or blood vessels. The current standard chemotherapy for patients with advanced PDA is gemcitabine. Gemcitabine can also be combined with nab-paclitaxel[5] or erlotinib[6], resulting in improved survival. Moreover, a multi-chemotherapy regimen (FOLFIRINOX) consisting of 5-fluorouracil, folinic acid, oxaliplatin and irinotecan has been associated with significant improvement in survival for patients with advanced PDA[7]. However, the currently used chemotherapeutic agents have still failed to demonstrate satisfactory clinical advantages in patients with advanced PDA. It has been well demonstrated that PDA is relatively resistant to chemotherapy, so new therapeutic strategies are urgently needed to improve pancreatic cancer treatment. Regarding potential targets for cancer vaccines, PDA cells express numerous tumor-associated antigens (TAAs), such as Wilms' tumor gene 1 (WT1)[8], mucin 1 (MUC1)[9], human telomerase reverse transcriptase (hTERT)[10], mutated K-Ras[11], survivin[12], carcinoembryonic antigen (CEA)[13], epidermal growth factor receptor 2 (HER-2)[14], and p53[15]. Therefore, cancer vaccines targeting these TAAs may be an alternative approach for treating patients with PDA.

INDUCTION OF ANTITUMOR IMMUNE RESPONSES

Cancer cells degrade endogenous antigens into short peptides (usually 8-10 amino acids) and present them *via* major histocompatibility complex (MHC) class Ⅰ molecules. These cells express numerous TAA-derived peptides on their cell surface as a result of malignant transformation. Meanwhile, T cells with the αβ T cell receptor (TCR) express CD4+ T cell or CD8+ T cell lineage markers[16]. Interaction of the TCR on CD8+ cytotoxic T lymphocytes (CTLs) with the complexes of antigenic peptides and MHC class Ⅰ molecules on tumor cells is a critical event in the T cell-mediated antitumor immune response. However, induction of CD8+ CTLs also requires antigenic peptides to be presented on the surface of antigen-presenting cells (APCs) in the context of MHC class Ⅰ molecules. It has become clear that dendritic cells (DCs) are the most potent APCs in the human body and play a pivotal role in the initiation, programming, and regulation of antitumor immune responses[17]. DCs can process endogenously synthesized antigens into peptides, which are presented on the cell surface as peptide/MHC class Ⅰ complexes, but require activation signals to differentiate and eventually migrate to the regional lymph nodes, where they are recognized by the αβ TCR on CD8+ T cells[17]. Moreover, DCs capture and process exogenous antigens and present peptide/MHC class Ⅰ complexes through an endogenous pathway *via* a process known as antigen cross-presentation[18]. This cross-presentation is essential for the initiation of $CD8^+$ CTL responses[19]. In contrast, exogenous antigens from the extracellular environment are captured and delivered to the compartments of the endosome/lysosome, where they are degraded into antigenic peptides, which are then complexed with MHC class Ⅱ and recognized by the αβ TCR of CD4+ T cells[17]. Finally, mature DCs can present TAAs to naive CD4+ and CD8+ T cells in the regional lymph nodes; these T cells then differentiate into activated T cells. It is well known that in the induction of efficient CD8+ CTL responses against cancer cells, CD4+ T cells are essential for the priming of CD8+ CTLs through activation of APCs and production of interleukin (IL)-2 and interferon (IFN)-γ[20]. CD4+ T cells also play an important role in the maintenance and infiltration of CD8+ CTLs at a tumor site[21]. Therefore, activation of antigen-specific CD4+ and CD8+ T cell responses by

Table 1 Cell-based cancer vaccines

Cell	Antigen source	Ref.
Dendritic cells	Whole tumor cell lysates	[33]
	MHC class Ⅰ restricted antigenic peptides	[33,56,60,61]
	MHC class Ⅰ and Ⅱ restricted antigenic peptides	[31,57,62]
	Dying or dead tumor cells	[34]
	mRNA encoding tumor associated antigens	[35,36]
	cDNA	[37]
	Exosomes	[38]
	Fusions generated with whole tumor cells	[39-43]
Immunogenic whole tumor cells	A GM-CSF-secreting, irradiated, allogeneic PDA cell line	[76-82]
Cancer stem cells	Cancer stem-like cell-associated antigens	[107-109]

GM-CSF: Granulocyte macrophage colony-stimulating factor; PDA: Pancreatic ductal adenocarcinoma; MHC: Major histocompatibility complex.

cell-based cancer vaccines, such as either DCs loaded with TAAs or modified whole tumor cells, is essential to induce efficient antitumor immunity against pancreatic cancer cells[22].

PDA cells can evade immune control through several mechanisms. One major mechanism is the immunosuppressive tumor microenvironment. The microenvironment in pancreatic cancer in particular consists of PDA cells and stroma cells, such as cancer-associated fibroblasts (CAFs), tolerogenic DCs, myeloid-derived suppressor cells (MDSCs), immunosuppressive tumor-associated macrophages (TAMs), and regulatory T cells (Tregs). Importantly, PDA cells themselves induce immune suppression through production of immunosuppressive substances such as cytokines [*e.g.*, transforming growth factor (TGF)-β, IL-10, and IL-6, vascular endothelial growth factor (VEGF), Fas ligand (Fas-L), programmed cell death-1 (PD-1) ligand (PD-L1) and indoleamine-2, and 3-dioxygenase (IDO)][22,23]. These immunosuppressive cells inhibit antitumor immunity by various mechanisms, including depletion of arginine and elaboration of reactive oxygen species (ROS) and nitrogen oxide (NO)[22,23]. The pancreatic cancer microenvironment not only contributes to pancreatic cancer-induced immune suppression but also might be closely related to the extent of disease. For example, T cells producing IL-22 were significantly increased in PDA tissue, and this increase was significantly associated with tumor staging and poor prognosis[24]. Moreover, Tregs, MDSCs, and T helper 17 (Th17) cells in intratumoral tissue elicited strong immune suppression in patients[25,26]. As a result, CD8+ CTL function in patients with advanced PDA is impaired by IL-10 and TGF-β from Tregs. Therefore, DC-based cancer vaccines against PDA cells that cause induction of TAA-specific CD4+ and CD8+ T cells combined with depletion of immunosuppressive cells may tip the balance in favor of immunostimulation.

DC-BSAED CANCER VACCINES

The aim of cancer vaccines is to induce efficient antitumor immunity. Peptide vaccines are frequently used because they are simple, safe, and economical. However, certain obstacles prevent the use of peptide vaccines from becoming widespread. The drawbacks of peptide vaccines are related to numerous factors: (1) the limited number of known synthesized short peptides cannot be presented *via* many MHC molecules[27]; (2) monoclonal CD8+ CTLs may be ineffective in reacting to PDA cells[28]; (3) certain TAAs and MHC class Ⅰ molecules are occasionally down-regulated, which may occur during tumor progression[28]; and (4) DCs may have impaired function in patients with advanced PDA[29]. Therefore, *in vitro*-generated mature DCs have been developed as cancer vaccines because of their powerful ability to induce antigen-specific CD4+ T cells and CD8+ CTL responses in preclinical and clinical studies[30]. To date, the majority of DC-based cancer vaccines have been generated using monocyte-derived DCs. Immature DCs can be generated by a single leukapheresis after culture in the presence of granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-4. In our laboratory, immature DCs are activated for vaccines by incubation with penicillin-killed and lyophilized preparations of a low-virulence strain (Su) of *Streptococcus pyogenes* (OK-432) and with prostaglandin E2 (PGE2), after which a large number of DCs can be cryopreserved in ready-for-use aliquots[31]. Several strategies have been used to develop DC-based cancer vaccines to elicit efficient antitumor immune responses (Table 1). To induce DC presentation of TAAs, DCs have been loaded with TAAs in the form of tumor lysates[32], antigenic peptides[33], dying or dead tumor cells[34], mRNA[35,36], cDNA[37], or exosomes[38] or have been fused with whole tumor cells to form hybrid cells[39]. The strategy of fusing DCs and whole tumor cells is based on the facts that DCs are potent APCs and that whole tumor cells express abundant TAAs, including both known and unidentified TAAs[40-42]. Therefore, DC-tumor fusion cells can process a broad array of TAAs and present them *via* MHC class Ⅰ and class Ⅱ in the context of co-stimulatory molecules[40-42]. Moreover, many adjuvants, including Toll-like receptor (TLR)3, TLR9, synthetic oligodeoxynucleotides (ODNs) containing unmethylated CpG, polyinosinic:polycytidylic acid (polyI:C), IL-2, IL-12, and IL-18, have been used in DC-based cancer vaccines to maximize antitumor immune responses in preclinical studies[43].

The field of cancer vaccines for PDA is currently in an active state of clinical investigation. In particular, the development of DC-based cancer treatments is of great importance. Clinical trials of DC-based cancer vaccines for PDA patients have been conducted (Table 2), including clinical trials for an MUC1-targeted DC-

Table 2 Clinical trials of dendritic cell-based cancer vaccines in pancreatic cancer patients

Cell-based cancer vaccines	Targets	Vaccines	Phase	Patients	Results	Ref.
Dendritic cells (DCs)	MUC1	DCs loaded with MUC1 peptide	Phase Ⅰ/Ⅱ	12 pancreatic or biliary cancer patients following surgical resection	These patients have been followed for more than 4 yr after vaccination, and 4 of them were alive without recurrence.	[46]
			Phase Ⅰ	16 patients with pancreatic cancer	2 of 15 patients with resected PDA were alive and disease free at 32 and 61 mo.	[47]
			Phase Ⅰ	7 patients with pancreatic cancer	These patients showed MUC1-specific immune responses; however, there was no significant clinical benefit.	[48]
		DCs transfected with MUC1 cDNA	Phase Ⅰ/Ⅱ	10 patients with pancreatic cancer	MUC1 specific immune responses were observed in 4 of 10 patients.	[49]
	WT1	DCs loaded with MHC class Ⅰ restricted WT1 peptides	Retrospective analysis	49 patients with pancreatic cancer refractory to standard treatment	The median survival time from vaccines was 360 d. Erythema reaction at the vaccination site was a prognostic factor for a significant survival benefit.	[56]
		DCs loaded with MHC class Ⅰ restricted WT1 peptides	Retrospective analysis	255 patients with pancreatic cancer refractory to standard treatment	The median survival time from diagnosis was 16.5 mo. Erythema reaction at the vaccination site was a prognostic factor for a significant survival benefit.	[60]
		DCs loaded with MHC class Ⅰ restricted WT1 peptides	Phase Ⅰ	10 patients with pancreatic cancer	The therapy was feasible, tolerable and effective in PDA patients without liver metastases.	[61]
		DCs loaded with MHC class Ⅰ and class Ⅱ restricted WT1 peptides	Phase Ⅰ	7 patients with pancreatic cancer	WT1 peptide-specific delayed-type hypersensitivity (DTH) was detected in 4 of 7 patients with PDA vaccinated with DC/WT1-Ⅰ/Ⅱ and in 0 of 3 patients with PDA vaccinated with DC/WT1-Ⅰ or DC/WT1-Ⅱ. All 3 PDA patients with strong WT1-specific DTH reactions had a median OS of 717 d. A patient with multiple liver metastases has remained alive for over 1000 d and received more than 71 vaccinations.	[31,62,63]
	hTERT	DCs transfected with hTERT mRNA	Phase Ⅰ	A patient who could not receive chemotherapy due to sever neutropenia	Vaccination was associated with induction of strong immune responses to multiple hTERT epitopes. The patient had been vaccinated with DC/hTERT mRNA alone for 3 yr and resulted in no evidence of active disease.	[66]
	CEA	DCs loaded with CEA mRNA	Phase Ⅰ	3 patients with resected pancreatic cancer following neoadjuvant vaccine therapy	All 3 PDA patients showed injection site reactivity and remained alive without recurrence at more than 2.5 yr from the original diagnosis	[68]
	DCs transfected with an adenovirus encoding IL-12		Phase Ⅰ	3 patients with pancreatic cancer	Intratumoral DC injections were guided by ultrasound. Vaccines induced a significantly increased infiltration of CD8+ T cells in some patients. A partial response was observed in 1 of 3 patients.	[73]
	penicillin-killed and lyophilized preparations of a low virulence strain (Su) of Streptococcus pyogenes (OK-432)-activated DCs and CD3-stimulated LAK cells		Phase Ⅰ	5 patients with pancreatic cancer	Intratumoral injection of OK432-activated DCs, followed by intravenous infusion of CD3-stimulated LAK cells. One patient had a partial response and 2 had stable disease for over 6 mo. The median OS was 478 d.	[75]
Peripheral blood mononuclear cells (PBMCs)	K-ras	irradiated PBMCs were used as antigen-presenting cells and loaded with K-ras peptide	Phase Ⅰ	9 patients with pancreatic cancer	Only one patient showed a positive cellular immune response. The worse prognosis of PDA patients on this immunization protocol using PBMCs as APCs may be associated with impaired induction of an antitumor immune responses.	[71]

MHC: Major histocompatibility complex; PDA: Pancreatic ductal adenocarcinoma; APC: Antigen-presenting cells; IL-12: Interleukin-12; WT1: Wilms' tumor gene 1; MUC1: Mucin 1; hTERT: Human telomerase reverse transcriptase; CEA: Carcinoembryonic antigen.

based cancer vaccination regimen. MUC1 is a TAA consisting of a polymorphic, glycosylated type Ⅰ transmembrane protein present in glandular epithelium and overexpressed in 90% of PDAs. Importantly, MUC1 is associated with poor prognosis, enhanced metastasis and chemoresistance[9,44]. It has been reported MUC1-

targeted cancer vaccines were effective in inducing antitumor immunity in murine pancreatic cancer models[45]. Therefore, several groups have conducted clinical trials with DCs loaded with MUC1 peptide (DCs/MUC1 peptide) or transfected with MUC1 cDNA (DCs/MUC1 cDNA). In a phase Ⅰ/Ⅱ clinical trial, following surgical resection, 12 patients with pancreatic or biliary cancer were vaccinated with MUC1 peptide-loaded DCs. These patients were followed for more than 4 years after vaccination, at which point 4 were alive and without recurrence[46]. In another phase Ⅰ study of 16 patients with PDA who were vaccinated with DCs/MUC1 peptide, 2 of 15 patients with resected PDA were alive and disease free at 32 or 61 mo[47]. Moreover, 7 PDA patients were vaccinated with DCs/MUC1 peptide in a phase Ⅰ trial[48]; these patients showed MUC1-specific immune responses, although there was no significant clinical benefit. MUC1-specific immune responses were also observed in 4 of 10 PDA patients following vaccination with DCs/MUC1 cDNA in a phase Ⅰ/Ⅱ trial[49]. Although the MUC1-targeted DC-based cancer vaccination regimen was safe and a significant MUC1-specific immune response was observed in several enrolled PDA patients, further investigation is warranted.

WT1

The WT1 antigen is also one of the most widely expressed TAAs in various tumor types, including PDA[50,51]. Importantly, WT1 has been ranked by the National Cancer Institute (NCI) as the number 1 target for cancer vaccines based on several factors: (1) therapeutic function; (2) immunogenicity; (3) the role of the antigen in oncogenicity; (4) specificity; (5) the expression level and percentage of antigen-positive cells; (6) stem cell expression; (7) the number of patients with antigen-positive cancers; (8) the number of antigenic epitopes; and (9) the cellular location of antigen expression[52]. WT1 has been found to be oncogenic, rather than tumor suppressive, in tumorigenesis[53]. Moreover, both cellular and humoral immune responses against the WT1 protein are naturally elicited in cancer patients, indicating that the *WT1* gene product is highly immunogenic[54,55]. Therefore, we and other groups have been performing clinical trials of the efficacy of WT1-targeted cancer vaccines for patients with PDA[31,56-63]. Four clinical reports about the use of DCs loaded with WT1 peptides combined with standard chemotherapy, such as gemcitabine, to treat advanced PDA patients have been published[31,56,60,61]. The vaccines can be mainly classified into 2 groups: (1) DCs loaded with MHC class I-restricted WT1 peptides (DC/WT1-I)[56,60,61] and (2) DCs loaded with multiple MHC class I- and class Ⅱ-restricted WT1 peptides (DC/WT1-I/Ⅱ)[31]. Both DC/WT1-I and DC/WT1-I/Ⅱ vaccinations are associated with significant induction of WT1-specific CD8+ T cells in circulating blood. In one study, Kobayashi *et al*[60] analyzed 255 PDA patients who received standard chemotherapy combined with DC-based cancer vaccines, including DC/WT1-I. The median survival time (MST) from diagnosis was 16.5 mo. Interestingly, an erythema reaction at the vaccination site was a prognostic factor for a significant survival benefit. DC/WT1-I-based cancer vaccines alone or combined with lymphokine-activated killer (LAK) cells were also retrospectively analyzed in 49 PDA patients[56]. Among all 49 patients, 2 had complete remission, 5 had a partial response, and 10 had stable disease. The survival of patients receiving DC-based cancer vaccines and standard chemotherapy (gemcitabine and/or S-1, an oral fluoropyridine) plus LAK cells was significantly longer than the survival of those receiving the vaccine in combination with chemotherapy but no LAK cells. Moreover, a prospective clinical trial using DC/WT1-I combined with gemcitabine demonstrated that the therapy was feasible, tolerable and effective in PDA patients without liver metastases[61]. We also conducted a phase Ⅰ study of chemoimmunotherapy using DC/WT1-I/Ⅱ vaccines and standard chemotherapy (gemcitabine and/or S-1) in 7 advanced PDA patients[31,57,62]. The combination therapy was well tolerated, and WT1-specific IFN-γ-producing CD4+ and CD8+ T cells were significantly increased following treatment with DC/WT1-I/Ⅱ. WT1 peptide-specific delayed-type hypersensitivity (DTH) was detected in 4 of the 7 patients with PDA who were vaccinated with DC/WT1-I/Ⅱ and in 0 of the 3 patients with PDA who were vaccinated with DC/WT1-I or DCs loaded with MHC class Ⅱ-restricted WT1 peptides (DC/WT1-Ⅱ). Moreover, the MST and the median progression-free survival (PFS) of the patients with PDA who were vaccinated with DC/WT1-I/Ⅱ were significantly longer than the MST and PFS of those receiving the DC/WT1-I or DC/WT1-Ⅱ vaccine. In addition, the WT1-specific DTH-positive patients who received DC/WT1-I/Ⅱ showed significantly improved overall survival (OS) and PFS compared with the negative-control patients. In particular, all 3 PDA patients with strong WT1-specific DTH reactions had a median OS of 717 d. Surprisingly, a patient with multiple liver metastases remained alive for more than 1000 d and received more than 71 vaccinations; this patient had strong WT1-specific DTH reactions throughout the vaccination period[63]. The combination of DC/WT1-I/Ⅱ and chemotherapy induced long-term WT1-specific CD4+ and CD8+ T cell responses. DC/WT1-I/Ⅱ may elicit not only effector but also long-lived effector memory and central memory T cells, all of which are capable of recognizing WT1-positive PDA cells and which are therefore associated with long-term stable disease[57].

hTERT

hTERT, the catalytic subunit of a functional telomerase complex, is also widely expressed in most human tumors and plays an essential role in tumor progre-

ssion[64]. Therapeutic strategies targeting such antigens involved in tumor growth resulted in antitumor immune responses in a mouse study[65]. As loss of telomerase activity may inhibit the progression of PDA cells, hTERT is a widely applicable target for triggering CTL responses. It was demonstrated that hTERT-specific immune responses were safely induced in a PDA patient vaccinated with DCs transfected with hTERT mRNA (DCs/hTERT mRNA)[66]. In this clinical study, DCs/hTERT mRNA vaccination was specifically administered to a PDA patient with relapsed disease[67]. The patient could not receive chemotherapy due to severe neutropenia and thus was vaccinated with DCs/hTERT mRNA alone for 3 years, which resulted in no evidence of active disease. The vaccinated patient also showed induction of strong immune responses to multiple hTERT epitopes. Therefore, hTERT-targeted DC-based cancer vaccines may be an effective approach for treating patients with PDA.

CEA

PDA cells widely express CEA, a glycosylated protein, so induction of CEA-specific immune responses may be associated with survival benefits[67]. In one clinical trial, 3 patients with resected PDA received neoadjuvant therapy, including DCs loaded with CEA mRNA (DCs/CEA mRNA), for 6 mo[68]. In this trial, all 3 PDA patients showed injection site reactivity and remained alive and without recurrence at more than 2.5 years from the original diagnosis. Although CEA-targeted cancer vaccinations induce strong CEA-specific immune responses, they usually fail to eradicate the tumor in most patients with advanced disease[67]. The results may be at least partly associated with the immunosuppressive effects of the tumor micro-environment. Therefore, to improve the clinical efficacy of CEA-targeted cancer vaccines, we need to design improved strategies that can overcome the immunosuppressive tumor microenvironment.

KRAS

As the *KRAS* gene is mutated in up to 95% of PDA cells[69], targeting mutant K-ras-specific immune responses may influence the clinical benefits of treatment for PDA patients[70]. To induce K-ras-specific antitumor immunity, irradiated peripheral blood mononuclear cells (PBMCs) were used as APCs and loaded with a K-ras epitope[71]. In this clinical trial, 9 patients with PDA, all with *KRAS* mutations, were vaccinated. Only one patient showed a positive cellular immune response, resulting in a median OS of 60 d. The worse prognosis of PDA patients subjected to an immunization protocol using PBMCs as APCs may be associated with impaired induction of antitumor immune responses per se. The vaccination protocol could be improved using mature DCs instead of PBMCs.

DCs combination therapy

The major cytokines currently in use or under evaluation for use in cancer vaccines are IFN-α, IL-2, GM-CSF, and IL-12[72]. An alternative strategy for clinical trials of DC-based cancer vaccines is use of IL-12-secreting DCs[73]. The main source of IL-12 in humans is DCs, and IL-12 acts as a major orchestrator of the T helper 1 (Th1)-type immune response against cancer when present directly in the tumor[74]. Therefore, 3 PDA patients were vaccinated with DCs transfected with an adenovirus encoding the IL-12 gene (DCs/IL-12)[73]. The intratumoral DC injections were mainly guided by ultrasound. DCs/IL-12 induced significantly increased infiltration of CD8+ T cells in certain patients, and a partial response was observed in 1 of the 3 patients with PDA[73]. As the DCs were not loaded with TAAs, cross-presentation of TAAs by the DCs in the patients must have been induced by IL-12. Another group reported administering gemcitabine and an endoscopic ultrasound-guided fine-needle injection of OK432-activated DCs into tumors in 5 PDA patients, followed by intravenous infusion of CD3-stimulated LAK cells[75]. Three of the 5 patients demonstrated effective responses: 1 had a partial response, and 2 had long-term stable disease for more than 6 mo[75]. The median OS was 478 d in this phase Ⅰ trial. In the patient with partial remission, induction of tumor antigen-specific CTLs was observed.

WHOLE TUMOR CELL-BASED CANCER VACCINES

Whole tumor cells can be genetically modified to produce cytokines to enhance antitumor responses. A GM-CSF-secreting, irradiated, allogeneic PDA cell line (GVAX) has been investigated in multiple phase Ⅰ and Ⅱ studies[76-82] (Table 3). GVAX recruits and activates DCs and promotes presentation of TAAs by DCs for activation of CD4+ and CD8+ T cells[83,84]. Early clinical trials demonstrated that vaccination with GVAX enhances CD8+ CTL responses against multiple mesothelin-specific epitopes that have been correlated with survival benefits[76-78]. As cancer vaccines alone have usually failed to demonstrate significant clinical activity in advanced PDA patients, PDAs are considered as non-immunogenic tumors, which is due to the immunosuppressive tumor microenvironment[80]. Recently, 39 PDA patients received GVAX alone or in combination with low-dose cyclophosphamide (Cy) to deplete Tregs[80]. Importantly, 33 of the 39 patients treated with GVAX showed the formation of vaccine-induced lymphoid aggregates. Moreover, the post-GVAX CTL infiltration and aggregate formation resulted in up-regulation of immunosuppressive regulatory mechanisms, including the PD-1/PD-L1 pathway. Therefore, GVAX-vaccinated PDA patients are better

Table 3 Clinical trials of whole tumor cell-based cancer vaccines in pancreatic cancer patients

Cell-based cancer vaccines	Vaccine	Phase	Patients	Results	Ref.
Whole tumor cell	GM-CSF-secreting allogeneic pancreatic cancer cell lines (GVAX) and chemoradiotherapy	Phase Ⅱ	14 patients with resected pancreatic cancer	3 patients were disease free at least 25 mo after diagnosis	[76]
	GVAX (arm A)/GM-CSF vaccine and cyclophosphamide (arm B)	Phase Ⅱ	50 patients with pancreatic cancer (2 arm)	Median OS: 2.3 mo in arm A, 4.3 mo in arm B	[77]
	GVAX and chemoradiotherapy	Phase Ⅱ	60 patients with resected pancreatic cancer	Induction of mesothelin-specific CD8+ T cells correlated with disease-free survival. Median OS: 24.8 mo	[78]
	Ipilimumab (anti-CTLA-4 monoclonal antibody) alone (arm 1), Ipilimumab and GVAX (arm 2)	Phase Ⅱ	30 patients with pancreatic cancer (2 arm: 1:1)	Three of 15 patients had evidence of prolonged disease stabilization (31, 71, and 81 wk) and 7 patients experienced CA19-9 declines (arm 1). In 2 of these patients, disease stabilization occurred after an initial period of progression. The median OS was 5.7 mo and 1 yr OS was 27%. Among patients with OS > 4.3 mo, there was an increase in the peak mesothelin-specific T cells and enhancement of the T-cell repertoire.	[79]
	GVAX	Phase Ⅱ	39 patients with pancreatic cancer	GVAX treatment was associated with the formation of vaccine-induced intratumoral tertiary lymphoid aggregates in 33 of 39 patients. Enhanced CD8+ CTL responses against multiple mesothelin-specific epitopes that have been correlated with survival benefits were also found.	[80]
	GVAX with low-dose cyclophosphamide (Cy) followed by CRS-207 (live-attenuated Listeria monocytogenes-expressing mesothelin) (arm A), GVAX + Cy (arm B)	Phase Ⅱ	90 patients with pancreatic cancer	Enhanced mesothelin-specific CD8+ CTL responses were associated with longer OS. Median OS was 9.7 mo (arm A, n = 61).	[81]
	Algenpantucel-L (2 pancreatic cancer cell lines that have been modified to express alpha-gal)	Phase Ⅱ	62 patients with resected pancreatic cancer	The 12-mo disease-free survival was 62%, and the 12-mo overall survival was 86%; the phase Ⅲ study is ongoing.	[82]

candidates for immune checkpoint therapies than vaccine-naive patients[79]. In a mouse study, a GVAX vaccine combined with anti-PD-1 antibody blockade improved murine survival compared with anti-PD-1 antibody or GVAX alone[85]. In a clinical trial, although GVAX alone also failed to show clinical benefits in PDA patients, infiltration of activated T cells expressing CTL-associated antigen 4 (CTLA-4) and PD-1 was induced by GVAX[80]. The efficiency of immune checkpoint-targeting agents is dependent on induction of adaptive immune responses[86]. Thus, they conducted combination therapy with inhibition of the CTLA-4 pathway using ipilimumab (anti-CTLA-4) and GVAX in metastatic PDA patients[79]. Three of 15 patients had evidence of prolonged disease stabilization (31, 71, or 81 wk), and 7 patients experienced a decline in carbohydrate antigen 19-9 (CA19-9). In 2 of these patients, disease stabilization occurred after an initial period of progression. The median OS was 5.7 mo, and 1-year OS was 27%. Among patients with OS > 4.3 mo, there was an increase in the peak mesothelin-specific T cell count and enhancement of the T cell repertoire[79]. Moreover, immunosuppressive pathways in the tumor microenvironment were overcome by the addition of the GVAX vaccine and low-dose Cy for PD-1 blockade. Therefore, combining anti-PD-1 or anti-PD-L1 antibody therapy with cancer vaccines such as GVAX may be effective therapy for PDA patients. In addition, they demonstrated that GVAX coupled with low-dose Cy followed by treatment with CRS-207 (live-attenuated *Listeria monocytogenes* expressing mesothelin) induced innate and adaptive immunity in 61 PDA patients. Mesothelin-specific CD8+ CTL responses enhanced by GVAX/Cy/CRS-207 were associated with longer OS (n = 61, 9.7 mo) compared with the responses enhanced by GVAX/Cy (n = 29, 4.6 mo)[81].

Whole tumor cells can be genetically modified to produce cytokines to inhibit tumor cell production of immunosuppressive cytokines, such as TGF-β, IL-10, IL-6, and VEGF. In particular, TGF-β has a critical role in immunosuppressive mechanisms, so down-regulation of TGF-β activates DCs and increases TAA-specific CTL induction. In mouse studies, several strategies to inhibit the production of TGF-β by cancer cells were developed. For example, TGF-β production by cancer cells was inhibited by the administration of neutralizing antibodies[87,88] and small interfering RNAs (siRNAs)[89] or constructs coding for a soluble variant of the TGF-β receptor[90]. We have previously demonstrated that the production of TGF-β, IL-10 and VEGF by human PDA cells is significantly limited upon exposure to

pharmaceutical-grade ethanol, without decreased expression of MHC class I and MUC1[91]. Therefore, whole tumor cells genetically modified to express immunosuppressive cytokines, such as GM-CSF, and to inhibit immunosuppressive cytokines, such as TGF-β, are better candidates for the generation of DC-based cancer vaccines for PDA patients.

CELL-BASED CANCER VACCINES COMBINED WITH CHEMOTHERAPY

Cytotoxic chemotherapy has been known to blunt immune responses because of the toxic effects of these treatments on dividing bone marrow progenitor cells, including lymphocytes. However, increasing evidence has suggested that cancer vaccines have the possibility of achieving better effects if combined with chemotherapy[92]. Cancer cells undergoing immunogenic apoptosis due to chemotherapy express calreticulin (CRT), which is a Ca2+-binding chaperone on the cell surface that mediates efficient phagocytosis by DCs[93,94]. In addition, high-mobility group box 1 (HMGB1)[95,96] and pentraxin-3 (PTX3)[97] are released from late-stage dying cancer cells to activate DCs and modulate immune responses *via* a TLR4-dependent signaling pathway. Therefore, necrotic or apoptotic tumor cells induced by chemotherapeutic agents enhance immunogenicity and can be effectively taken up by DCs, resulting in efficient processing of TAAs for presentation to T cells. For example, a standard cytotoxic agent for PDA, gemcitabine, can enhance the cross-presentation of TAAs by DCs as well as CTL induction[98]. Moreover, Cy and gemcitabine can each augment the antitumor effects by depleting immunosuppressive cells such as Tregs, B cells and MDSCs as well as by inducing the proliferation of DCs, all of which potentially enhances the antitumor immune response[98-101]. We also reported that up-regulated presentation of WT1 peptide *via* MHC class I molecules on PDA cells is induced by exposure of the cells to gemcitabine and/or S-1[102]. Importantly, WT1-specific CTLs can more efficiently lyse gemcitabine-treated PDA cells than untreated cells[102]. Certain TAAs that are not usually expressed on cancer cells may be uncovered by treating cancer cells with chemotherapeutic agents; these antigens are good targets for cancer vaccines because they can be effectively recognized by antigen-specific CTLs[103]. Therefore, cancer vaccines can synergize with chemotherapy in targeting PDA cells[104]. In addition, our recent reports indicate that the combination of gemcitabine and trastuzumab conjugated to a cytotoxic agent (T-DM1) may be a promising modality for the treatment of PDA cells with low human epidermal growth factor 2 (HER2) expression as a result of the unique HER2-up-regulating effect of gemcitabine[105]. Importantly, cancer patients who have previously received cancer vaccines could also benefit more from subsequent chemotherapy than those patients who are not vaccinated[106].

Although conventional treatments such as chemotherapy can eradicate certain cancer cells, the remaining cancer stem cells (CSCs) can lead to tumor relapse. Although CSCs have been implicated in chemoresistance, these remaining CSCs are still attractive targets for cancer vaccines[107,108]. Therefore, it is desirable to develop a novel therapy that selectively targets CSCs *via* cancer vaccines, which can be combined with conventional chemotherapy. Indeed, expression of TAAs such as MUC1 is up-regulated in CSCs by chemotherapy, and CSCs are efficiently lysed by MUC1-specific CTLs[108,109]. CSC-loaded DC-based cancer vaccines may be an alternative approach. We have reported that DCs fused with CSC cells induced CSC-specific CD4+ and CD8+ T cells with high production of IFN-γ, which is predominantly produced by Th1 cells[108]. Therefore, developing surgery/chemotherapy targeting the bulk of cancer cells combined with cell-based cancer vaccines targeting CSCs is highly desirable.

CONCLUSION

CTLA-4 and PD-1 are well-described co-inhibitory molecules that are highly expressed by TAAs-specific CTLs and associated with impaired antitumor immune responses. In contrast, PD-L1, which binds to PD-1, is not constitutively expressed in tumor cells but is induced in response to IFN-γ produced by activated T cells[110]. Therefore, immune checkpoint inhibitors, such as CTLA-4, PD-1 and anti-PD-L1 antibody, may be an efficient means for treating cancer patients[110]. Indeed, antibodies can be used to block inhibitory ligand/receptor interactions by acting on certain cancer cells (*e.g.*, anti-PD-L1) or T cells (*e.g.*, anti-CTLA-4 or anti-PD-1), allowing enhancement of the antitumor immune response in specific tumors[111]. However, single-agent immune checkpoint inhibitors, such as CTLA-4, PD-1, and anti-PD-L1 antibody, elicit limited adaptive immune responses in PDA patients due to the non-immunogenic tumor microenvironment, which provides a formidable barrier to CTL infiltration at baseline[85]. Therefore, cell-based cancer vaccines may prime PDA patients for treatment with better candidate checkpoint inhibitors[112]. Combining a blockade of multiple inhibitory pathways with cell-based cancer vaccines may synergistically decrease T cell anergy and improve clinical benefits.

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Combined hepatocellular cholangiocarcinoma: Controversies to be addressed

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Abstract

Combined hepatocellular cholangiocarcinoma (CHC) accounts for 0.4%-14.2% of primary liver cancer cases and possesses pathological features of both hepatocellular carcinoma and cholangiocarcinoma. Since this disease was first described and classified in 1949, the classification of CHC has continuously evolved. The latest definition and classification of CHC by the World Health Organization is based on the speculation that CHC arises from hepatic progenitor cells. However, there is no evidence demonstrating the common origin of different components of CHC. Furthermore, the definition of CHC subtypes is still ambiguous and the identification of CHC subtype when a single tumor contains many components has remained unresolved. In addition, there is no summary on the newly recognized histopathology features or the contribution of CHC components to prognosis and outcome of this disease. Here we provide a review of the current literature to address these questions.

Key words: Progenitor cell origin; Pathology classification; Combined hepatocellular cholangiocarcinoma

Core tip: This review article focuses on the current views about the histopathology and clinical characteristics of combined hepatocellular cholangiocarcinoma (CHC). Whether the different components of CHC share a common cell origin is still ambiguous. Furthermore, the definition of CHC subtype is still ambiguous and

the identification of CHC subtype when a single tumor contains many components has remained unresolved. The features between hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC) make CHC has better prognosis than CC but poorer than HCC.

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INTRODUCTION

Combined hepatocellular cholangiocarcinoma (CHC) is a rare form of primary liver cancer with pathological features of both hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC). CHC accounts for 0.4%-14.2% of primary liver cancer cases in different regions[1-4]. It was first described and classified by Allen and Lisa in 1949[5], and Goodman updated the pathology classification in 1985[3]. Currently, according to the World Health Organization (WHO), CHC is classified into the classical subtype and subtype with stem-cell features[6].

Despite the extensive study on CHC over the past 60 years, many questions remain unresolved. Although many studies demonstrated that CHC originated from progenitor cells[7-9], whether all subtypes of CHC with stem-cell features share a common origin is unknown. Furthermore, how to classify the newly recognized histopathology features and how many effects on survival for CHC of the diversity of components and the properties of each component remain unclear. In addition, how to distinguish CHC form CK19(+)HCC and how to identify the subtype when a single tumor contains many components are still ambiguous. Finally, the clinical features of CHC including the risk factors, the role of liver transplantation and diagnosis are still controversial.

Therefore, here we review the current literature on CHC to address these issues.

EVOLUTION OF CHC CLASSIFICATION

Allen and Lisa first described CHC in 1949[5]. The authors classified this type of tumor into three subtypes including (1) separate masses composed of either HCC or CC; (2) contiguous but independent masses of HCC and CC; and (3) an intermingling of hepatocellular and glandular elements. In 1985, Goodman[3] updated the classification as (1) collision tumors, a coincidental occurrence of both HCC and CC in the same patient; (2) transitional tumors including areas of intermediate differentiation and an identifiable transition between HCC and CC; and (3) fibrolamellar tumors, which resemble the fibrolamellar variant of HCC but also contain mucin-producing pseudoglands. In 1989, The Liver Cancer Study Group of Japan[10] formulated its own classification of CHCs into three types: double cancer, combined type, and mixed type. In 1994[11], CHC was universally defined by the WHO as a tumor with intimate and unequivocal mixtures of both HCC and CC cells. However, these tumors should be distinguished from cases with separate HCC and CC arising in the same liver and in which HCC and CC are present at adjacent locations.

With the development of medicine, an increasing number of studies have demonstrated that hepatic progenitor cells play an important role in the development of CHC. Therefore, in 2010, the WHO updated the classification of CHC into the classical type and subtypes with stem cell features[6]. The subtypes were further subdivided into the typical subtype, intermediate cell subtype, and cholangiolocellular subtype. The classical type of CHC contains unequivocal components of HCC and CC, whereas subtypes with stem cell features possess special histopathology features (Table 1). This most recent classification system has been widely adopted.

Origin

Since the identification of CHC occurred, the debate on the origin of CHC has been ongoing. It is theoretically reasonable for CHC to originate from hepatocytes, cholangiocytes or hepatic progenitor cells. Many published studies have aimed to validate the origin of CHC at genetic and protein levels.

Genetic level

Multiple studies have been conducted to verify whether the HCC and CC components of CHC share the same origin and to identify the origin of the components. In 1996, Imai *et al*[12] investigated alterations of p53, K-ras and Rb-1 genes in seven CHC patients. The authors found that both components of CHC have the same genetic characteristics and speculated that they thus share the same origin. In 2000, Fujii *et al*[13] studied the allelic status of chromosome arms 1p, 1q, 3p, 4q, 5q, 6q, 8p, 9p, 10q, 11q, 13q, 16q, 17p, 17q, 18q, and 22q in HCC and CC foci of CHC. According to genetic patterns, they classified CHC origin into three possibilities: (1) a collision tumor in which two independent neoplastic clones develop at close proximity; (2) a single clonal tumor with divergent potential; and (3) a single clonal process in which genetic heterogeneity occur in the process of clonal evolution. This was the first unique classification of the origin of CHC at the genetic level. The authors considered that different types of CHCs had the same or different origins. Cazals-Hatem[14] screened for loss of heterozygosity (LOH) and for p53 and beta-catenin

Table 1 Evolution of classification of combined hepatocellular cholangiocarcinoma

Ref.	Classification
Allen *et al*[5], 1949	Separate masses
	Contiguous but independent masses
	Intimate intermingling of hepatocellular and glandular element
Goodman *et al*[3], 1985	Collision tumors, a coincidental occurrence of both HCC and CC in same patient
	Transitional tumors including areas of intermediate differentiation
	Fibrolamellar tumors, having features of fibrolamellar HCC and CC
Liver Cancer Study Group of Japan[10] 1989	Double cancer
	Combined type
	Mixed type
WHO[6] 2010	CHC-classical: typical HCC and typical CC
	CHC-SC
	CHC-SC-typical: nests of mature looking hepatocytes with peripheral clusters of small cells that have a high nucleus:cytoplasm ratio and hyperchromatic nuclei
	CHC-SC-int: tumor cells show features intermediate between hepatocytes and cholangiocytes. These tumor cells show strands, solid nests and/or trabeculae of small, uniform cells with scant cytoplasm and hyperchromatic nuclei
	CHC-SC-CLC: admixtures of small monotonous glands, antler-like anastomosing patterns. Each tumor cell is cuboidal, smaller in size than normal hepatocytes, with a high nucleus: cytoplasm ratio, and distinct nucleoli

CHC: Combined hepatocellular-cholangiocarcinoma; CHC-SC-typical: Combined hepatocellular-cholangiocarcinoma, stem cell features, typical subtype; CHC-SC-int: Combined hepatocellular-cholangiocarcinoma, stem cell features, intermediate cell subtype; CHC-SC-CLC: Combined hepatocellular-cholangiocarcinoma, stem cell features, cholangiolocellular subtype; HCC: Hepatocellular carcinoma; CC: Cholangiocarcinoma.

mutations in 9 CC, 15 CHC and 3 collision tumors compared with 137 HCC cases. Recurrent specific LOH was identified at 3p and 14q with significant differences among CHC, CC and HCC. The authors concluded that CHC is genetically more similar to CC compared with HCC. CHC shared frequent +1q (71%), +8q (57%), and 8p (57%) with HCC and higher numbers of imbalance with CC. The similar chromosomal changes with HCC and CC suggested that both share characteristics of CHC. These data also supported the same origin of different components of CHC. Although the genetic studies of CHC have provided some preliminary findings, whether HCC and CC components within CHC share the same origin should be further explored.

Protein level

Currently, the widely accepted origin of CHC is hepatic progenitor cells. However, it really takes a long time to get the conclusion. Many studies have used immunohistochemistry analyses to evaluate protein expression in CHC. A study by Okada *et al*[15] in 1987 reported the same levels of ABH, Lewis, and sialyl Lea antigens in CHC and HCC, indicating that CHC might have a hepatocellular origin. Imai *et al*[12] studied the expression patterns of carcinoembryonic antigen and keratin in seven CHC patients and demonstrated the same phenotypic characteristics in both components of CHC, suggesting a common origin of the hepatocellular and cholangiocarcinoma components. Zhang *et al*[8] performed immunohistochemical analysis of twelve CHC cases for hepatocytic (hepPar1, alpha-fetoprotein), cholangiocytic cytokeratin (CK7, CK19), hepatic progenitor cell (OV-6), hematopoietic stem cell (c-kit, CD34), as well as CD45 and chromogranin-A markers. The results suggested that CHC has the same hepatic progenitor cell origin. The same conclusion was also obtained by Akiba *et al*[16] who performed immunohistochemical analyses of biliary markers (CK7, CK19, and EMA), hepatocyte paraffin (hepPar-1), hepatic progenitor cell markers[17] (CD56, c-kit, CD133, and EpCAM), and vimentin. Kim *et al*[18] studied the expression of Yes-associated protein 1 (YAP1), a potential oncogene that can promote stem cell proliferation, among three groups comprising 36 HCCs with stem characteristics, 64 HCCs without stemness and 58 CHCs. Higher expression of YAP1 was observed in CHCs and HCCs with stemness than in HCCs without stemness. From the results of the expression of hepatocellular, cholangiocellular and progenitor cell markers, we speculate that the origin of CHC is hepatic progenitor cell.

UNSOLVED HISTOPATHOLOGY PROBLEMS

Although the classification of CHC has been widely accepted, there are still some special histopathology features that have not been classified. Nakajima[19] first described CHC with sarcomatous transformation in 1988, and several similar cases have since been reported[20-22]. In 2013, Terada[23] reported a CHC case with ductal plate malformation features that was characterized with CC cells forming markedly irregular tubules with intraluminal cell projections, bridge formations, and intraluminal tumor biliary cells. Jung *et al*[24] reported cholangiocellular carcinoma with satellite nodules showing intermediate differentiation.

Current studies have established that CHC contains various components. However, which component plays a more important role in the prognosis of CHC and whether the number of components could affect the recurrence and survival remain ambiguous. Akiba *et al*[16] investigated 54 CHC cases according to the WHO classification. The pathology type was defined by predominant histologic pattern (≥ 50%). CHC has wide histologic diversity, which poses a challenge for classification of CHC. Ikeda *et al*[25] divided 24 CHC cases into two groups: group A with less than 5% stem cell areas, and group B with more than 5% stem cell features. The expression level of delta-like 1 homolog was higher in group B than in group A. The postoperative overall survival rate was better in group A than in group B. These results suggest that the contribution of different components in CHC might be a significant factor that affects outcome. Sasaki *et al*[26] examined 62 CHC patients and found that the intermediate cell subtype was significantly associated with gender, tumor size, and histological grade of HCC and inversely correlated with the degree of stromal fibrosis. Significant associations were observed between cholangiocellular carcinoma and degree of fibrosis and inflammation, and an inverse association was observed with histological grade of HCC. The proportion of typical subtype was significantly inversely correlated with the degree of inflammation. Furthermore, the histological diversity score was also associated with vascular invasion. These data demonstrate a correlation between the proportion of each stem cell subtype and the histological diversity with clinicopathological factors, suggesting various properties of each component in the development of CHC. Although the WHO provided a clear definition and classification of CHC, the roles of newly recognized histopathology features, properties of each component, and the functions of diversity of components in recurrence and survival still need further exploration. In addition, the definition of CHC subtypes is still ambiguous, and how to distinguish CHC from CK19(+)HCC and how to identify the subtype of CHC when a single tumor contains many kinds of components require further research.

CLINICAL CHARACTERISTICS

Risk factors

Similar to other primary liver cancers, CHC could be induced by various factors impairing liver parenchyma. Compared with HCC, the relationship between HBV or HCV and CHC incidence is relatively weak[27]. A Japanese study revealed that the anti-HCV-positive rate was high in CHC as well as in HCC[28]. A hospital-based case-control study in China found that HBV infection and heavy alcohol intake might contribute to the development of CHC[29]. The percent of HCV-HBC infection was 37.3% and chronic liver disease was 38.3%, which suggested that viral infection and cirrhosis may be risk factors for ICC and CHC.

Clinical features

Whether the clinical features of CHC are similar to those of HCC or CCC is controversial. Compared with CC, the clinicopathological features of CHC include more advanced histological differentiation, increased prevalence in males, and lower levels of serum bilirubin and ALP. These features may make it possible to diagnose CHC in patients with suspected CC[30]. CHC is a hypovascular liver cancer with striking elevation of serum AFP and multiple regional lymphadenopathy[31]. These features are similar to both HCC and CC. The prevalence of hepatitis B positivity and cirrhosis in CHC was intermediate between HCC and CC[2,32]. CHCs were more likely to occur in males than CC[33]. Compared with HCC, CHC had lower incidence in Asian or Pacific patients with less distant spread. While clinical characteristics of CHC are similar to those of HCC, overall survival is more similar to that of ICC. Ng *et al*[34] investigated 21 cases of CHC and found that invasive characteristics with venous permeation, direct invasion into liver parenchyma and microsatellite formation were similar to those of HCC. The hypovascular features and regional lymphadenopathy features of CHC resemble those of CC. The elevations of serum AFP, venous permeation, and direct invasion and microsatellite formation in CHC are similar to those of HCC. The 75% positive rate of hepatitis B surface antigen and 61.5% AFP elevation rate detected in CHC were also closer to levels in HCC[34]. The features of both HCC and CC make CHC be a typical cancer having no special characters.

DIAGNOSIS

Imaging methods

The development of imaging methods has led to their important role in evaluating the properties of primary liver cancer. Dynamic computed tomography for CHC diagnosis has three enhancement patterns, including type Ⅰ hyper-enhancement in the early phase and hypo-enhancement due to washout of contrast medium in the late phase, resembling HCC; type Ⅱ peripheral enhancement in the early phase; and type Ⅲ late phases and an area of hyper-enhancement in the early phase and an area of slight delayed enhancement in the late phase. Type Ⅲ can be identified on the presentation of computed tomography[35]. Contrast-enhanced computed tomography could also predict the dominant component of CHC, which could optimize the treatment strategy for CHC patients[36]. Sensitivities and specificities for diagnosis of CHC range from 33% to 34% and 81% to 100%, respectively[37]. Therefore, tumor markers and risk factors should be used to improve the accuracy of diagnosis. Magnetic resonance imaging also contributes to the diagnosis of CHC[4].

Pathology

Before operation, it is difficult to accurately diagnose CHC. Although it is not entirely possible to confirm reliable diagnosis of CHC on cytologic preparations alone, cellblock or core biopsy for histochemical and immunohistochemical studies could be helpful for diagnosis[38,39]. Analysis of operative specimens is the gold standard for the diagnosis of CHC.

Treatment

Although the prognosis of CHC is poor, the development of hepatectomy, liver transplantation, and adjuvant chemotherapy and radiation therapy has improved the survival of patients.

Hepatectomy is the most important treatment for CHC, and can prolong the life of patients and even cure disease. Eguchi *et al*[40] reported successful hepatectomy for the intrahepatic recurrence of a CHC case and showed that resection of recurrent tumors could improve the poor prognosis of CHC.

Liver transplantation is another treatment for CHC. In CHC patients with liver transplantation, the 5-year survival was better than liver transplantation for intrahepatic CC but poorer than that of HCC meeting the Milan criteria[41-44]. The overall survival rates of CHC patients with liver transplantation at 1, 3 and 5 years were 79%, 66% and 16%, respectively[43]. Groeschl *et al*[45] showed that the survival benefit for localized CHC transplantation is similar to liver resection for CHC, but inferior to transplantation for HCC. Itoh *et al*[46] showed that better survival could be achieved after the living-donor liver transplantation for CHC meeting the Milan criteria or the Kyushu University criteria, similar to that of HCC.

Apart from operation, several adjuvant treatments are available, such as chemotherapy and radiation therapy. Disease-free survival of 42 mo after operation for a CHC case with lymph node metastasis was achieved through adjuvant chemotherapy and radiation therapy[47]. The case suggested an improved prognosis using multimodal therapy for CHC. Oral administration of UFT[48] effectively treated CHC with lymph node metastases. Systematic chemotherapy with fluorouracil, doxorubicin and cisplatin effectively suppressed the progression of CHC[49].

Together these findings suggest that for treatment of CHC, we should combine surgery with multimodal therapy to improve the survival of patients.

PROGNOSIS

The prognosis of primary liver cancer is poor. How about the prognosis of CHC? Tickoo investigated 27 CHCs with regard to their clinical features, which were different from those of pure HCC[50]. CHC showed disappointing prognosis, with overall 3- and 5-year survival rates of 30% and 18%, respectively. The 1- and 3-year survival rates in the CHC group (81.9% and 47%, respectively) were higher than those of the CC group and lower than those of the HCC group, which suggested a better prognosis of CHC compared with CC but worse compared with HCC[2]. In another center[4], the overall survival rates of CHC at 1, 3 and 5 years were 53%, 26% and 12%, respectively. Thus, the survival of CHC appears to be better than that of CC but worse than that of HCC[51]. Many factors could affect recurrence and survival after treatment. In a study of 30 CHC patients, Lin *et al*[4] found that major vascular branch invasion, regional organ invasion, nodal and distant metastases could affect prognosis. Park *et al*[52] concluded that the presence of portal vein thrombosis, distant metastasis and cholangiocellular component might be a poor prognosis indicator. Age, gender, transarterial chemoembolization and T stage were significant prognosis factors in CHC patients. The prognosis is poorer once the cholangiocellular components occur in liver cancer[52]. Sex, tumor-related factors (tumor number, major thrombus, and microvascular thrombus), serum gamma-glutamyl transpeptidase (GGT), and carbohydrate antigen 19-9 level seemed to be independent prognosis factors of long-term surgical survival for HBV-related CHC[53]. Disease-free survival could be affected by tumor size, major thrombus and serum GGT. The overall survival for CHC was associated with tumor size and lymph node metastasis. Poorer disease-free survival and similar overall survival rates were observed between CHCs and CCs[54]. The presence of cirrhosis, percent of serum hepatitis B or C marker positivity and the level of serum alpha-fetoprotein may be prognosis factors. Together these data indicate that CHC has a poor prognosis and many factors affecting the survival of HCC and CC could also be important prognostic factors for CHC.

Prospective

The obvious features between HCC and CC make CHC have better prognosis than CC but poorer than HCC. Owing to the limited therapy, the improvement of prognosis could be achieved by the development of basic science. With the development of sequencing techniques, it will soon be possible to demonstrate whether the different components of CHC share the same origin at the DNA level[55]. In addition, we could further explore whether CHC originates from hepatic progenitor cells at the RNA and protein levels. Finally, to address the unsolved pathology questions of CHC, many more studies are needed to clearly distinguish CHC from CK19(+)HCC and define the correct subtypes when a single mass has many different components. Owing to the variation of function in deciding the survival of different cancer components, perhaps a score system on the properties of each component and the numbers of components to predict the prognosis

could be established.

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ORIGINAL ARTICLE

Basic Study

Epithelial-to-mesenchymal transition in pancreatic ductal adenocarcinoma: Characterization in a 3D-cell culture model

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Abstract

AIM: To analyze the effect of three-dimensional (3D)-arrangement on the expression of epithelial-to-mesenchymal transition markers in pancreatic adenocarcinoma (PDAC) cells.

METHODS: HPAF-Ⅱ, HPAC, and PL45 PDAC cells were

cultured in either 2D-monolayers or 3D-spheroids. Ultrastructure was analyzed by transmission electron microscopy. The expression of E-cadherin, β-catenin, N-cadherin, collagen type I (COL-I), vimentin, α-smooth muscle actin (αSMA), and podoplanin was assayed by confocal microscopy in cells cultured on 12-mm diameter round coverslips and in 3D-spheroids. Gene expression for E-cadherin, Snail, Slug, Twist, Zeb1, and Zeb2 was quantified by real-time PCR. E-cadherin protein level and its electrophoretic pattern were studied by Western blot in cell lysates obtained from cells grown in 2D-monolayers and 3D-spheroids.

RESULTS: The E-cadherin/β-catenin complex was expressed in a similar way in plasma membrane cell boundaries in both 2D-monolayers and 3D-spheroids. E-cadherin increased in lysates obtained from 3D-spheroids, while cleavage fragments were more evident in 2D-monolayers. N-cadherin expression was observed in very few PDAC cells grown in 2D-monolayers, but was more evident in 3D-spheroids. Some cells expressing COL-I were observed in 3D-spheroids. Podoplanin, expressed in collectively migrating cells, and αSMA were similarly expressed in both experimental conditions. The concomitant maintenance of the E-cadherin/β-catenin complex at cell boundaries supports the hypothesis of a collective migration for these cells, which is consistent with podoplanin expression.

CONCLUSION: We show that a 3D-cell culture model could provide deeper insight into understanding the biology of PDAC and allow for the detection of marked differences in the phenotype of PDAC cells grown in 3D-spheroids.

Key words: Epithelial-to-mesenchymal transition; E-cadherin; 3D-spheroids; Podoplanin; Pancreatic ductal adenocarcinoma

Core tip: The functions of living tissue can be mimicked by three-dimensional (3D) cell cultures, thereby providing a method of decoding the information encoded in the tissue architecture. We aimed to analyze the effect of 3D-arrangement on the expression of some key markers of epithelial-to-mesenchymal transition in pancreatic adenocarcinoma (PDAC) cells cultured in either 2D-monolayers or 3D-spheroids. Our results show that a 3D-cell culture model could provide deeper insight into understanding the biology of PDAC and allow for the detection of marked differences in the phenotype of PDAC cells grown in 3D-spheroids.

Gagliano N, Celesti G, Tacchini L, Pluchino S, Sforza C, Rasile M, Valerio V, Laghi L, Conte V, Procacci P. Epithelial-to-mesenchymal transition in pancreatic ductal adenocarcinoma: Characterization in a 3D-cell culture model. *World J Gastroenterol* 2016; 22(18): 4466-4483 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i18/4466.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i18.4466

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive and lethal tumors, representing the fourth most common cause of cancer death in the Western world, with an estimated incidence of more than 40000 cases per year in the United States. The 5-year survival for all stages of the disease remains < 5%[1,2], due to the high incidence of recurrence and metastases dissemination[3].

During carcinogenesis, the "phenotypic switch" of pancreatic epithelial cells to mesenchymal cells, the so-called "epithelial-to-mesenchymal transition" (EMT), plays a pivotal role in PDAC progression, rendering tumor cells invasive and able to metastasize distant organs[4]. The EMT-related phenotype is characterized by the loss of epithelial features, including cell adhesion and polarity, following down-regulation of E-cadherin, cytoskeleton reorganization by expressing vimentin and α-smooth muscle actin (αSMA), and the motile properties and secretion of matrix metalloproteinases (MMPs)[5]. Several inducers of EMT transcription factors have been described, such as Snail, Slug, Twist, and Zeb, repressing E-cadherin expression *in vivo* and in various cancer cell lines, including lung, breast, colorectal, and ovarian cancer, thus inducing tumor malignancy[6-8]. It was demonstrated that Snail and Slug could increase invasion of breast, squamous, and pancreatic cancer cells[9-12]. The loss of E-cadherin is known to be a pivotal event, although experimental evidence demonstrates that 6 out of 7 PDAC commercial cell lines maintain E-cadherin expression in the cell membrane. Moreover, the similar expression of EMT markers in PDAC and benign pancreatic ducts[13] increases the relevance of studies aimed at definitively clarifying the role of EMT in PDAC development and progression, with particular attention paid to the expression of E-cadherin.

It is generally recognized that plastic or glass substrates commonly used for cell culture are not representative of the cellular environment found in organisms. Cells cultured as monolayers do not reproduce the structural organization or functional differentiation of the epithelium *in vivo*[14], and sometimes signaling pathways are fundamentally differently regulated than in polarized structures[15]. *In vitro* three-dimensional (3D) culture systems reduce the differences between 2D cell cultures and physiological tissues, thereby offering the possibility of investigating aspects of tumor biology and pathophysiology by maintaining a 3D cancer cell arrangement that reflects the *in vivo* tissue and tumor situation in relation to cell-cell interaction and differentiation patterns[16]. Therefore, 3D cultures, such as the well-established spheroid culture system,

could better reflect the *in vivo* behavior of cells in tumor tissues[17].

As PDAC remains currently one of the most lethal cancers, comprehension of its biology, development, and progression remains crucial for making inroads into this devastating human disease. The aim of this study was to investigate the expression of the main EMT markers in HPAF-Ⅱ, HPAC, and PL45 PDAC cell lines, grown in either 2D-monolayers or 3D-spheroids. Our goal was to use 3D cultures to bridge the gap between traditional cell cultures and *in vivo* settings with a method that mimics the 3D structure of living tissue in order to better characterize the phenotype of PDAC cells and, therefore, their behavior. We were particularly interested in understanding if the expression of E-cadherin is affected by these two different cell arrangements, in order to obtain new information on the effective role of this marker in PDAC.

MATERIALS AND METHODS

2D-monolayer cell culture and 3D-spheroid preparation

Three human pancreatic cancer cell lines (HPAF-Ⅱ, HPAC, and PL45) from pancreatic ductal adenocarcinoma (PDAC) (American Type Culture Collection, ATCC) were studied. PDAC cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mmol/L glutamine, antibiotics (100 U/mL penicillin, 0.1 mg/mL streptomycin), and 0.25 μg/mL amphotericin B. Cell viability was determined by trypan blue staining.

To obtain 3D-spheroids, PDAC cells (5×10^4 cells) were seeded in 24-well multiwell plates coated with 1% agarose in DMEM.

Spheroid integrity was verified by phase-contrast imaging after 3 d, 1 wk, and 2 wk, and cell viability in 3D-spheroids was determined by calcein fluorescence. For this purpose, 3D-spheroids were incubated with calcein-AM (3 μg/mL in PBS) for 30 min at 37 ℃, 5% CO_2 and observed under a fluorescence inverted microscope. In live cells, the nonfluorescent calcein AM is converted to green-fluorescent calcein after acetoxymethyl ester hydrolysis by intracellular esterases. For morphological and molecular evaluations, spheroids were harvested after 10 d. Duplicate samples of PDAC cells grown in 2D-monolayers and 3D-spheroids were analyzed.

To understand the invasive behavior of 3D-spheroids, HPAF-Ⅱ spheroids were seeded in basement membrane extract (BME) (Geltrex, Life Technologies), following the manufacturer's instructions. Single 3D-spheroids were suspended in 2% BME in complete DMEM, and then seeded in a 96-well multiwell plate coated with a thick layer of BME. 3D-spheroids were observed under an inverted microscope at different time points and monitored for a 14 d period to detect whether or not they were able to invade the surrounding environment.

Ultrastructural characterization by transmission electron microscopy

HPAF-Ⅱ, HPAC, and PL45 cells were grown as 2D-monolayers on Petri dishes. At confluence, cells were fixed with a solution containing 2% freshly prepared paraformaldehyde and 2% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.4). 3D-spheroids were harvested and fixed in the same fixative. Both 2D-monolayer cultures and 3D-spheroids, after fixation for 2-4 h at 4 ℃, were rinsed twice in cacodylate buffer for 20 min, post-fixed in 1% osmium tetroxide in the same buffer at 0 ℃ for 30 min, washed in distilled water, and stained *en bloc* with 2% aqueous uranyl acetate. After dehydration in graded ethanols, 2D-monolayer cultures (*in situ* on Petri dishes) and 3D-spheroids were embedded in Epon-Araldite resin.

Semi-thin sections 0.5 μm thick were stained with 0.5% toluidine blue in 1% sodium borate and examined using a light microscope (Zeiss Axiophot) for preliminary observations. Ultra-thin sections cut by a Leica Supernova ultramicrotome were stained with lead citrate and observed under a Zeiss EM10 electron microscope.

Immunofluorescence and confocal microscopy

HPAF-Ⅱ, HPAC, and PL45 cells were cultured on 12-mm diameter round coverslips into 24-well culture plates. When at the desired confluence, cells were washed in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde in PBS containing 2% sucrose for 10 min at room temperature, post-fixed in 70% ethanol, and stored at -20 ℃ until use. 3D-spheroids were fixed for 3 h in the same conditions. Cells grown in 2D-monolayers and 3D-spheroids were then washed in PBS three times and incubated overnight at 4 ℃ with the primary antibodies anti-E-cadherin (1:2500, Becton Dickinson), anti β-catenin (1:500, Novocastra), anti-N-cadherin (1:200, Santa Cruz), anti-collagen type Ⅰ (COL-I) (1:2000, Sigma Aldrich), anti-vimentin (1:200, Novocastra), anti-αSMA (1:400, Sigma Aldrich), and anti-podoplanin (15 μg/mL, Sigma Aldrich). Secondary antibodies conjugated with Alexa 488 (1:500, Molecular Probes, Invitrogen) were applied for 1 h at room temperature in PBS containing 25 μmol/L rhodamine-phalloidin in PBS and 0.2% triton X-100 in the dark. Negative controls were incubated that omitted the primary antibody. Finally, cells on coverslips and 3D-spheroids were incubated for 15 min with DAPI (1:100.000, Sigma Aldrich) and mounted onto glass slides using Mowiol. PDAC cells grown in 2D-monolayers or 3D-spheroids were analyzed by confocal microscopy (Olympus FV1000).

Real-time PCR

Total RNA was isolated by a modification of the acid guanidinium thiocyanate-phenol-chloroform method (Tri-Reagent, Sigma, Italy). One μg of total RNA was reverse-transcribed in 20 μL final volume of reaction

mix (Bio-Rad, Segrate-Milan, Italy). mRNA levels for E-cadherin, Snail, Slug, Twist, Zeb1, and Zeb2 were assessed. GAPDH was used as an endogenous control to normalize the differences in the amount of total RNA in each sample. The primer sequences were as follows: GAPDH: sense CCCTTCATTGACCTCAACTACATG, antisense TGGGATTTCCATTGATGACAAGC; E-cadherin: sense GAACGCATTGCCACATACAC, antisense GAATTCGGGCTTGTTGTCAT; Snail: sense CTTCCA GCAGCCCTACGAC, antisense CGGTGGGGTTG AGGATCT; Slug: sense TGTTTGCAAGATCTGCGGC, antisense TGCAGTCAGGGCAAGAAAAA; Twist: sense AGCAAGATTCAGACCCTCAAGCT, antisense CCTGGT-AGAGGAAGTCGATGTACCT; Zeb1: sense GAAAGTCAT-CCAGCCAAATGG, antisense ACTTGGTTCTCAGC TTGGGGAATCA; and Zeb2: sense GCTACACGTTTTGC-CTACCGC, antisense CGATTACCTGCTCCTTTGGGTT.

Amplification reactions were conducted in a 96-well plate in a final volume of 20 μL per well containing 10 μL of 1× SYBR Green Supermix (Bio-Rad, Italy), 2 μL of template, and 300 pmol of each primer. Each sample was analyzed in triplicate in a Bioer LineGene 9600. The cycle threshold (Ct) was determined and gene expression levels relative to that of GAPDH were calculated.

Western blot

Cell lysates were prepared in Tris-HCl 50 mmol/L pH 7.6, 150 mmol/L NaCl, 1% Triton X-100, 5 mmol/L EDTA, 1% SDS, proteases inhibitors, and 1 mmol/L sodium orthovanadate. Lysates were incubated on ice for 30 min and centrifuged at 14000 *g*, for 10 min at 4 ℃ to remove cell debris. Cell lysates (40 μg of total proteins) were diluted in SDS-sample buffer, loaded on 10% SDS-polyacrylamide gel, separated under reducing and denaturing conditions at 80 V according to Laemmli, and transferred at 90 V for 90 min to a nitrocellulose membrane in 0.025 mol/L Tris, 192 mmol/L glycine, 20% methanol, and pH 8.3. After electroblotting, the membranes were air dried and blocked for 1 h.

For E-cadherin evaluation on cell lysates, membranes were incubated for 1 h at room temperature in monoclonal antibody to E-cadherin (1:2500, Becton Dickinson) and, after washing, in HRP-conjugated rabbit anti-mouse serum (1:40000 dilution, Sigma, Italy). To confirm equal loading, membranes were reprobed by monoclonal antibody to α-tubulin (1:2000 dilution, Sigma Aldrich). Immunoreactive bands were revealed using the Amplified Opti-4CN or the Opti-4CN substrate Bio-Rad.

Analysis of results

Data are expressed as mean ± SD. Since the number of samples in each experimental group was low, we did not analyze our data by inferential statistics; rather, we aimed to measure differences between cells grown in 2D-monolayers or 3D-spheroids by calculating the effect size according to Cohen[18].

RESULTS

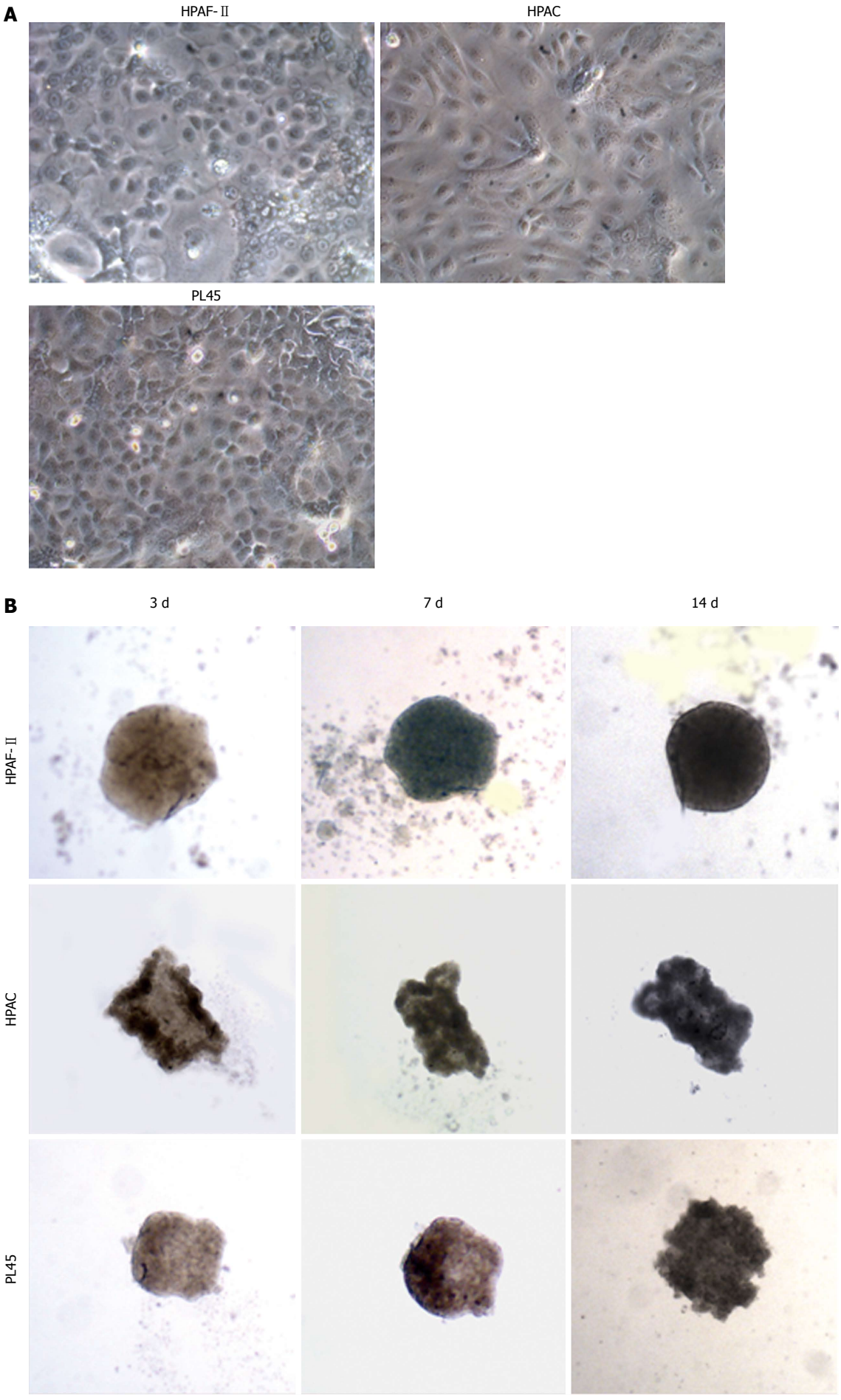
3D-spheroid morphology and viability

When seeded in agarose-coated wells, PDAC cells formed 3D aggregates that were evident after 40-72 h. HPAF-Ⅱ, HPAC, and PL45 cells cultured in 2D-monolayers were characterized by an epithelial morphology (Figure 1A). Inverted microscope observation of the 3D-spheroids at different time points showed that, after one week, cell density was increased and the spheroid exhibited a compact structure, with different morphologies in different cell types (Figure 1B). The size of the 3D-spheroids was approximately 300-500 μm. No evident differences were observed after two weeks, and so the spheroids were harvested for molecular and morphological evaluations after 10 d. To assess eventual necrosis in the inner part of the spheroids, possibly due to reduced delivery of nutrients, cells were stained with calcein-AM. Observations *via* fluorescent microscope revealed that all the cells were metabolically active, as they were all fluorescent (Figure 1C). Cell integrity in 3D-spheroids was also confirmed by transmission electron microscopy (TEM) (Figures 2-4).

Ultrastructural characterization by transmission electron microscopy

HPAF-Ⅱ cultured in 2D-monolayers grew as flat layers in which light or small dark cells partially overlapped and arranged with the basolateral membrane adhering to the plastic dish. Cells contained organelles and showed apical microvilli facing the culture medium (Figure 2A). HPAF-Ⅱ 3D-spheroids showed a compact structure consisting of multiple cell layers, with microvilli extensively covering the apical surfaces of the outer cellular layer (Figure 2B-D). Cells had variable shapes and cytoplasm amount, and sometimes contained mucin granules and small autophagic vacuoles. They also frequently exhibited a pale and markedly segmented nucleus (Figure 2B), with adjacent cells in the outer layer connected by junctional complexes located in the apical-lateral domain and by numerous interdigitations and desmosomes in their lateral domains (Figure 2C and D), which was consistent with cell polarity. The inner part of 3D-spheroid adjacent cells were separated by an evident intercellular space and linked by interdigitations. Additionally, some cells were arranged to delimit lumen-like structures (Figure 2B).

HPAC cells appeared as flat layers of light and dark cells that only partially overlapped and were interdigitated and connected by junctions in some cases. They exhibited short microvilli on the surface facing the culture medium. Cells contained organelles, autophagosomes, and showed other morphological similarities with HPAF-Ⅱ (Figure 3A). HPAC grown in 3D-spheroids showed a compact organization consisting of multicellular layers (Figure 3B). Epithelial cells of the outer layer presented their apical domain towards the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HPAC
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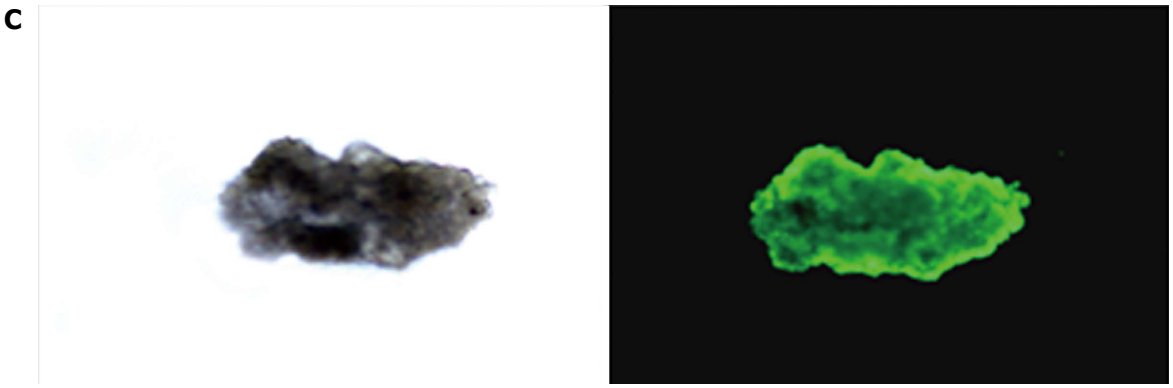


Figure 1 Morphology of pancreatic adenocarcinoma cells grown in 2D-monolayers and 3D-spheroids. A: Micrograph from inverted microscope showing the epithelial morphology of HPAF-Ⅱ, HPAC, and PL45 cells grown in 2D-monolayers. Original magnification: 20 ×; B: 3D-spheroids observed under inverted microscope after 3, 7, and 14 d. HPAF-Ⅱ spheroids were more rounded and uniformly dense; by contrast, HPAC and PL45 spheroids displayed an irregular shape. Original magnification: 10 ×; C: Representative 3D-spheroid under inverted microscope and after incubation with calcein-AM. Original magnification: 10 ×.

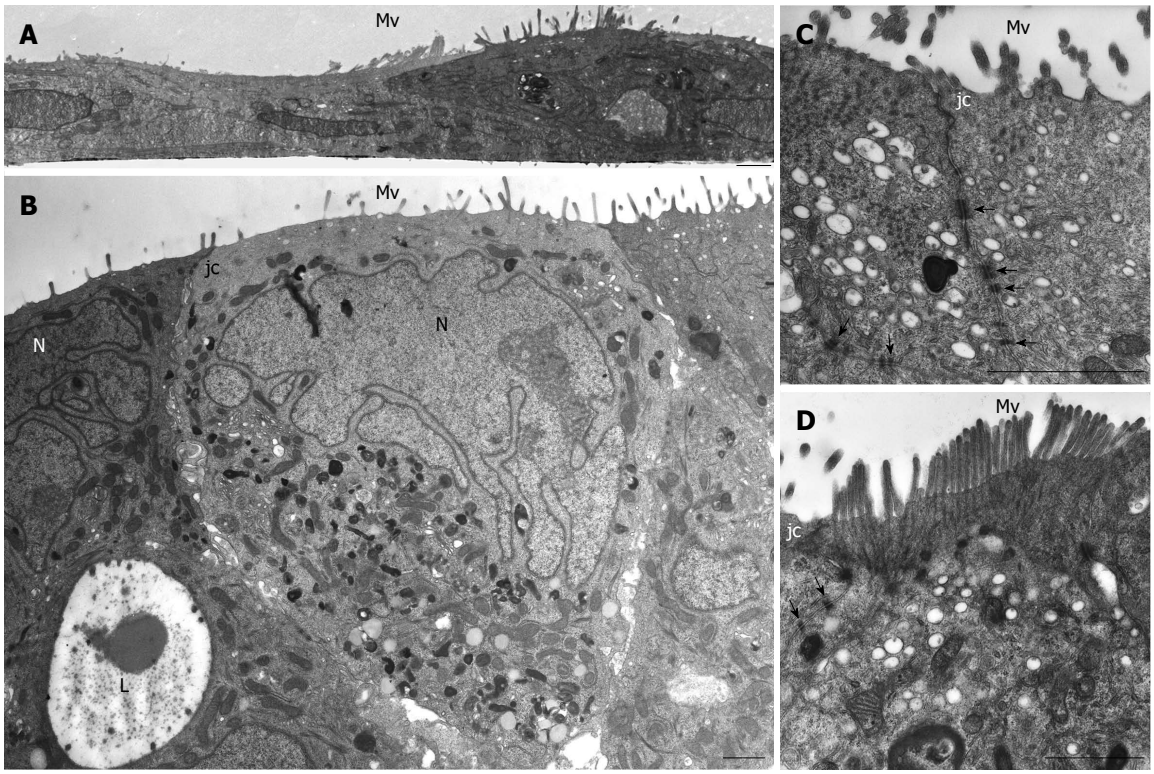


Figure 2 HPAF-Ⅱ ultrastructure. A: Electron microscopy of HPAF-Ⅱ culture grown in a 2D-monolayer showing two cells partially overlapping and exhibiting microvilli (Mv). Scale bar = 1 μm; B-D: Ultrastructural features of HPAF-Ⅱ cells grown in 3D-spheroids. Adjacent cells located at the periphery of the spheroid show microvilli (Mv) in the apical domain and junctional complexes (jc). In B, some cells exhibit a pale and markedly irregular nucleus (N). Some adjacent cells delimit a lumen-like structure (L). Scale bar = 1.5 μm. C, D**:** Adjoined cells are connected by junctional complexes (jc) in their apical-lateral domains and by numerous adherens junctions (arrows) in their lateral domains. In D, microvilli are densely packed; at their core, actin microfilaments can be seen extending into the apical cytoplasm. C, D: scale bar = 1.5 μm.

culture medium and were extensively covered with microvilli (Figure 3B2-D). Cell polarity was also evident as Golgi complexes and numerous mitochondria were present in the apical compartment (Figure 3B2 and C); moreover, the cytoplasm occasionally exhibited mucin granules at the apical pole (Figure 3C). Nuclei were located at the opposite side and characterized by widely dispersed chromatin and sometimes by a markedly irregular shape (Figure 3C). HPAC cells in 3D-spheroids exhibited a junctional complex consisting of tight junctions (zonulae occludentes), adherens junctions (zonulae adhaerentes), and desmosomes (maculae adhaerentes) in the apical-lateral domains (Figure 3D). In the internal region of 3D-spheroids, adjacent cells were separated by a more evident intercellular space and were interdigitated with neighboring cells by finger-like projections, without any evident junctional specializations (Figure 3B2). Notably, according to

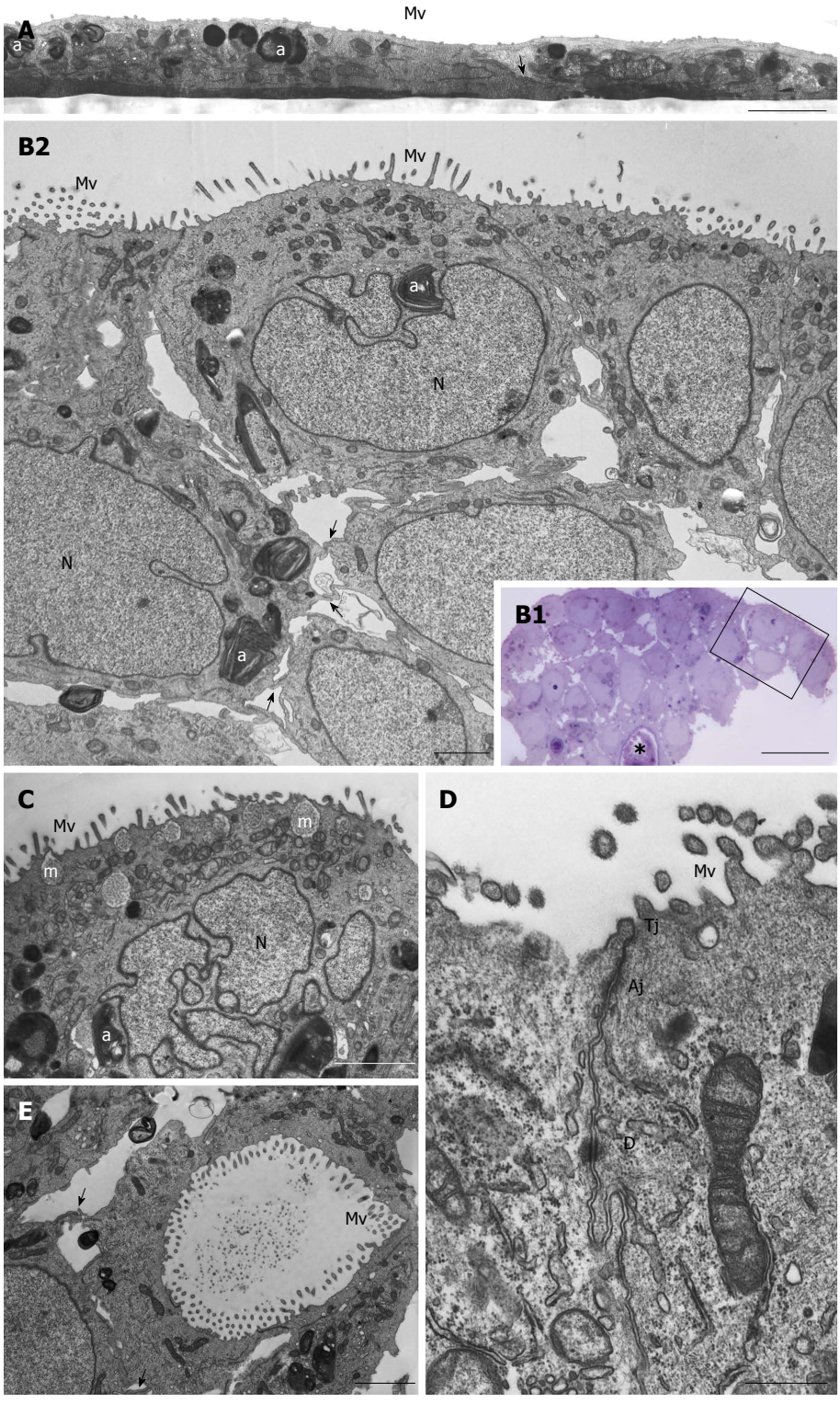


Figure 3 HPAC ultrastructure. A: Electron microscopy of a HPAC 2D-monolayer. Two cells having dark and light cytoplasm, respectively, are partially overlapped and interdigitated by finger-like projections (arrow); sparse and short microvilli (Mv) in the domain facing the culture medium and some autophagosomes (a) can be seen. Scale bar = 2 μm; B1: Light microscopy of a semi-thin section showing a representative area of a multilayered 3D-spheroid. The asterisk indicates a lumen-like structure. Ultrastructural features of the boxed area are shown in B2. Scale bar = 20 μm; B2: Electron microscopy of the thin section immediately adjacent to the semi-thin one, and corresponding to the boxed area in B1, shows cellular polarity and numerous microvilli (Mv) in the apical domain facing the culture medium. Several autophagosomes (a) can be observed in the cytoplasm. Cells of the lower region exhibit interdigitating finger-like processes (arrows) in the interstitial space. Nuclei (N) are euchromatic and frequently display more or less deep invaginations. Scale bar = 5 μm; C: Micrograph showing a cell with a markedly irregular nucleus (N) and several mucin granules (m) in the apical cytoplasm. Scale bar = 2 μm; D: Thin section of two adjacent cells located at the periphery of the spheroid and facing the culture medium. Microvilli (Mv) in the apical domain and a junctional complex, consisting of tight junction (Tj), adherens junction (Aj), and desmosome (D) in the lateral domain, can be observed. Scale bar = 0.5 μm; E: Micrograph of the inner part of a spheroid in which some adjacent interdigitated cells (arrows) delimiting a lumen-like structure exhibit numerous microvilli in their apical domains and junctional specializations (arrows). Scale bar = 2 μm

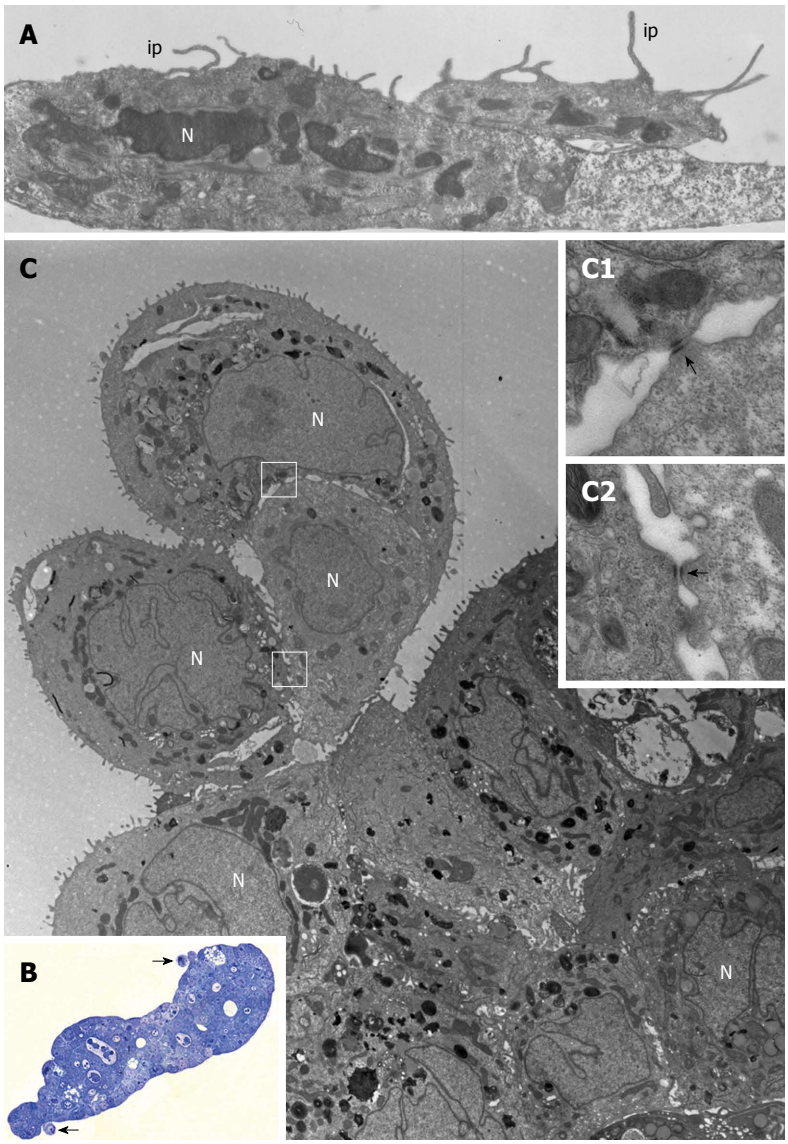


Figure 4 PL45 ultrastructure. A: Electron microscopy of two PL45 cells grown in 2D-monolayers that exhibit protrusions consistent with invadopodia (ip) originating from their surface and projecting toward the culture medium. N: Nucleus. Scale bar = 1 μm; B: Light microscopy of a semi-thin section showing a multilayered spheroid with 2 small groups of cells partially detached from the periphery of the spheroid (arrows). Scale bar = 40 μm; C: Electron microscopy of a thin section adjacent to the semi-thin one showing a group consisting of 3 cells joined to each other. The outlined areas are shown at greater enlargement in inserts C1 and C2; arrows indicate desmosomes. N: Nucleus. Scale bar = 5 μm. Inset scale bar = 0.25 μm.

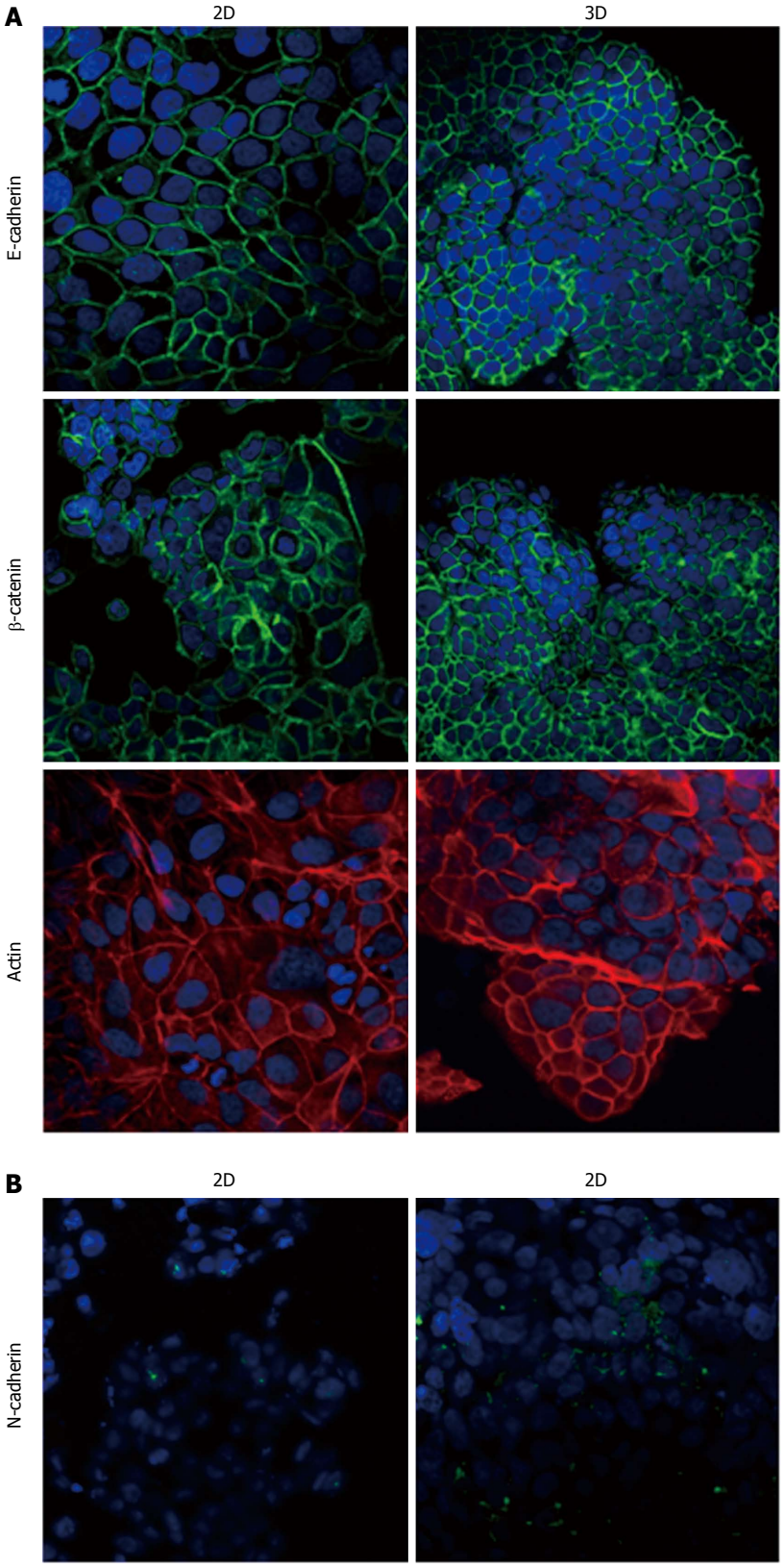
the description of elevated basal autophagy of PDAC cells, numerous autophagosomes were present in the cytoplasm of both the outer and inner regions of 3D-spheroids (Figure 3B2 and C)[19]. In the internal part of HPAC spheroids, some adjoined cells were linked by junctional specializations and arranged in lumen-like structures which appeared polarized with numerous microvilli projecting into the lumen (Figure 3E).

PL45 cells cultured in 2D-monolayers showed a light or dark cytoplasm in a similar manner to HPAF-Ⅱ and HPAC cells. However, unlike HPAF-Ⅱ and HPAC grown in 2D-monolayers, some cells showed protrusions of the plasma membrane similar to invadopodia of about 6 μm in length that projected toward the culture medium (Figure 4A).

PL45 grown in 3D-spheroids were arranged in multiple layers (Figure 4B and C) and frequently had markedly irregular nuclei (Figure 4C). In the cytoplasm, lipid droplets, autophagosomes, and numerous mitochondria were mainly located close to the nucleus. Interestingly, small groups of cells linked to each other by desmosomes (Figure 4C; inserts C1 and C2) seemed partially detached from the peripheral area of the spheroid (Figure 4B and C).

E-cadherin and β-catenin expression, and actin arrangement

Immunofluorescence analysis revealed that E-cadherin and β-catenin were strongly expressed at cell boundaries in both PDAC 2D-monolayers and 3D-spheroids, suggesting the presence of functional adherens junctions (Figures 5-7). In 2D-monolayers, actin filaments were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Actin
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N-cadherin

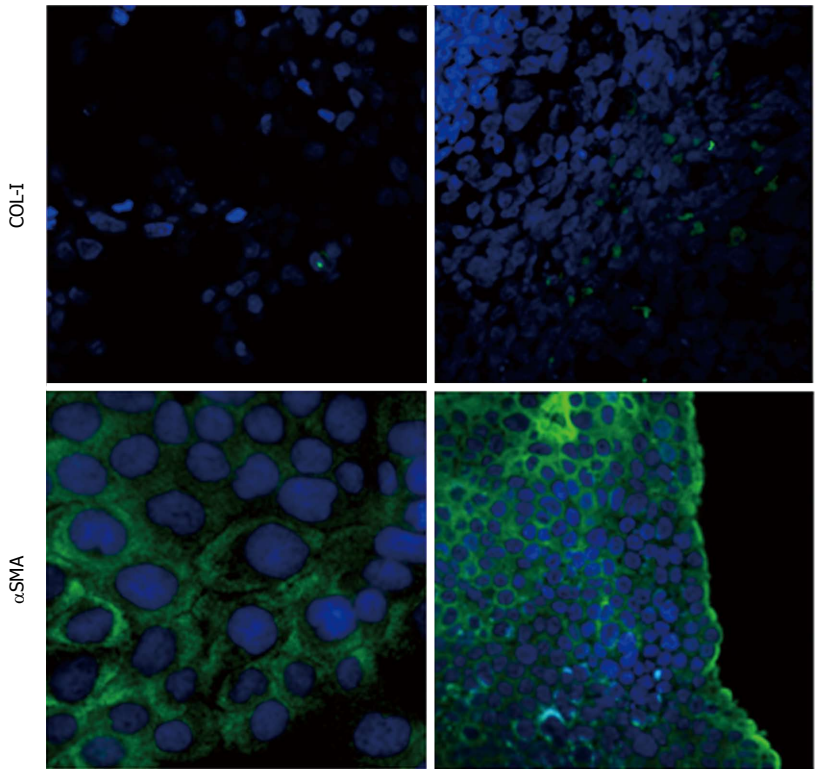


Figure 5 Expression of epithelial-to-mesenchymal transition-related markers in HPAF-Ⅱ cells. Micrographs using a confocal microscope showing epithelial (A) and mesenchymal markers (B) in HPAF-Ⅱ cells grown in 2D-monolayers and 3D-spheroids. Original magnification: 60 ×.

arranged just beneath the plasma membrane, forming the cortical actin, although actin fibers were also found in the cytoplasm. In HPAC and PL45, the presence of actin fibers was even more evident, suggesting focal adhesions which attach to the plastic substrate. In contrast, cortical actin filaments were much more evident in 3D-spheroids, as observed in differentiated epithelial cells (Figures 5A, 6A, and 7A). Gene expression analysis revealed that E-cadherin mRNA levels were highly expressed in 2D-monolayers compared to 3D-spheroids. This difference was evident since the effect size was > 2 (5.68 and 7 in HPAC and PL45 cells, respectively) (Figure 8A). In contrast, Western blot analysis showed that full-length E-cadherin (120 kDa) was expressed to a higher extent in 3D-spheroids (Figure 8B and C). In fact, lysates of HPAF-Ⅱ, HPAC, and PL45 cells grown in 3D-spheroids contained higher E-cadherin expression when compared to the relative 2D-monolayers (effect size: 6.15, 6.91, and 36.28, respectively), which was consistent with stronger cell adhesion. Moreover, in cells grown in 2D-monolayers, lower full-length E-cadherin levels were paralleled by an increase in E-cadherin degradation fragments that was consistent with cell junction disruption and lower cell adhesion. The molecular weight of some E-cadherin fragments were consistent with CT1, CT2, and CT3, as previously described[20,21].

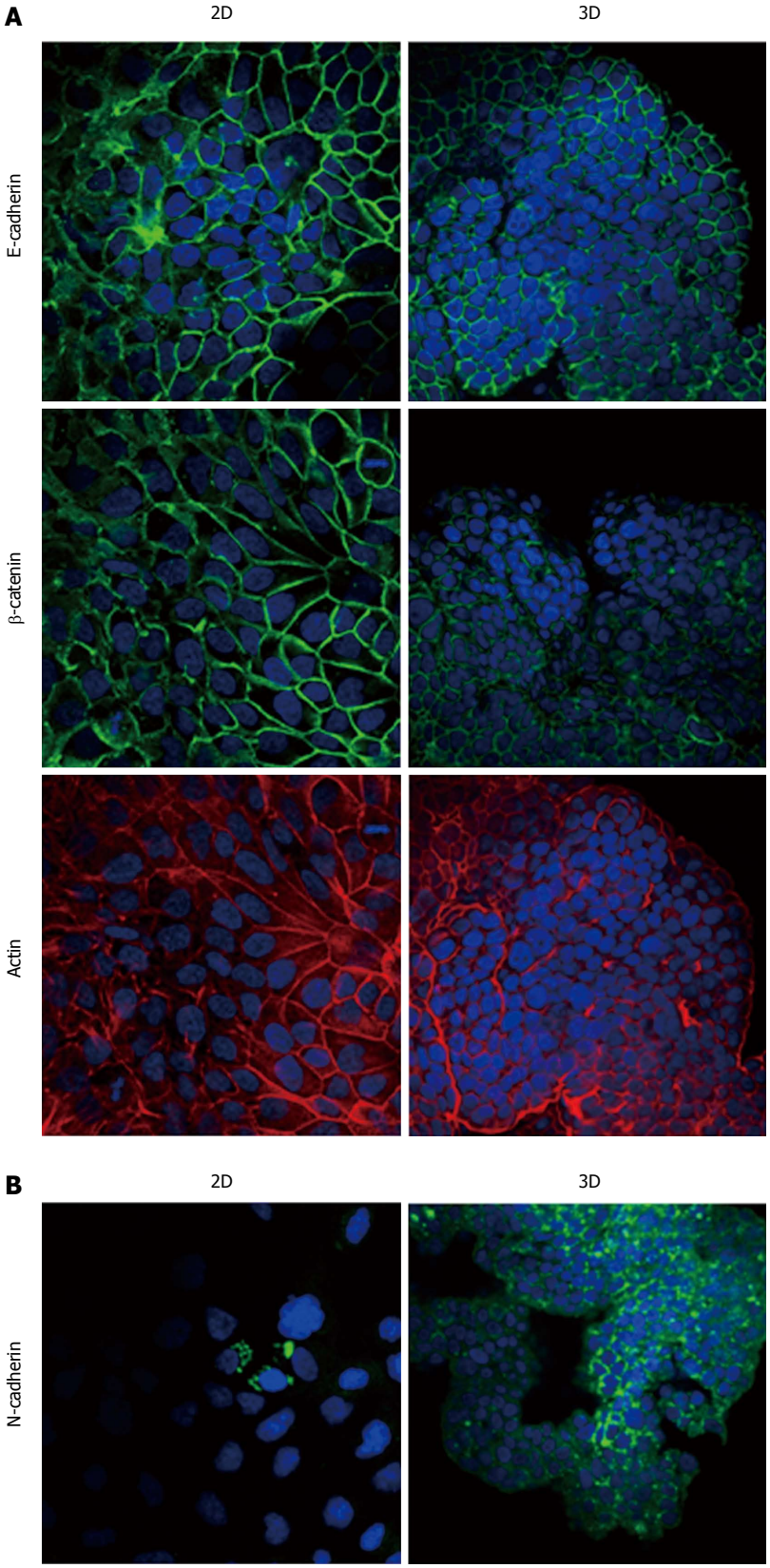
Mesenchymal-related phenotype marker expression

Using immunofluorescence, the expression of EMT markers was analyzed with respect to the acquisition of a mesenchymal phenotype suggestive of EMT. N-cadherin was almost undetectable in PDAC cells grown as 2D-monolayers and immunoreactivity was mostly diffuse in the cytoplasm. N-cadherin immunoreactivity was more frequent in 3D-spheroids, mostly in the cytoplasm. In HPAC spheroids, N-cadherin was sometimes detected at cell boundaries, which was consistent with the presence of functional adherens junctions containing this "mesenchymal" transmembrane protein. Although COL-I was expressed by very few scattered PDAC cells grown in 2D-monolayers, its expression seemed more frequent in 3D-spheroids, particularly in HPAC cells (Figures 5B, 6B, and 7B). αSMA was evident in both experimental conditions (Figures 5B, 6B, and 7B) in the three considered cell lines. Vimentin was undetectable (data not shown).

The EMT markers Twist, Snail, Zeb1, and Zeb2 were almost undetectable in both experimental conditions (data not shown). Slug was expressed at very low levels in HPAF-Ⅱ, as well as in PL45 2D-monolayers and 3D-spheroids, and was detected in HPAC at a higher degree in cells grown in 2D-monolayers compared to HPAC in 3D-spheroids (Figure 8D). Low or undetectable mRNA levels of these EMT markers were consistent with a high expression of E-cadherin under both experimental conditions.

Podoplanin expression and 3D-spheroid matrix invasion

Podoplanin was expressed in the cytoplasm of PDAC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Actin
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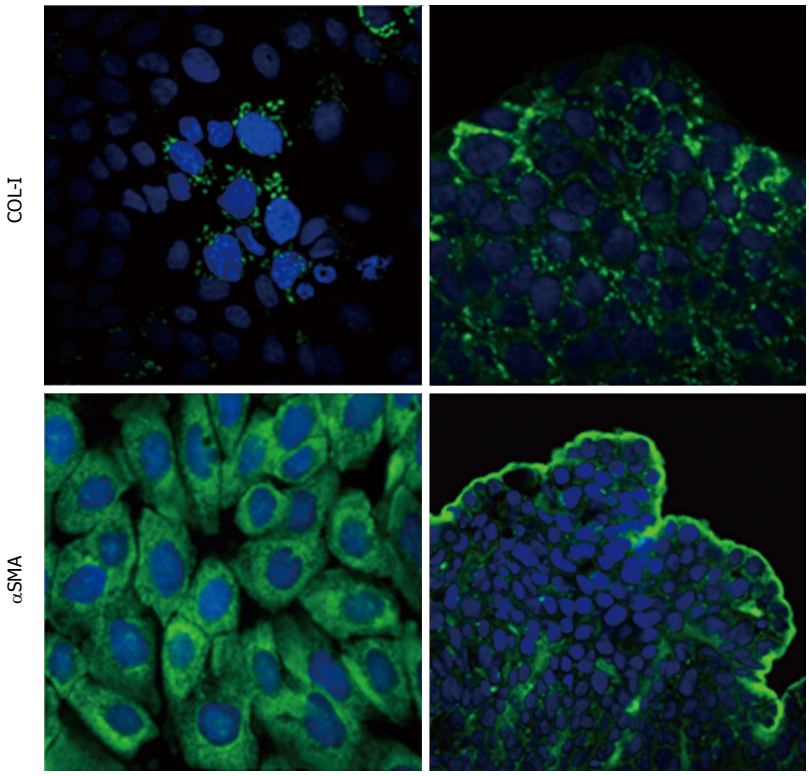


Figure 6 Expression of epithelial-to-mesenchymal transition-related markers in HPAC cells. Micrographs using a confocal microscope showing epithelial (A) and mesenchymal markers (B) in HPAC cells grown in 2D-monolayers and 3D-spheroids. Original magnification: 60 ×.

cells grown in either 2D-monolayers or 3D-spheroids (Figure 9A). Since podoplanin expression was described in cells undergoing collective migration, HPAF-Ⅱ 3D-spheroids were placed in BME to monitor their morphology during invasion of the surrounding matrix. We did not observe any evident spindle-like projections extending in the BME, but small clusters of cells did seem to detach from the spheroid surface to invade the surrounding environment (Figure 9B). These findings were confirmed by TEM analysis. In these experimental conditions we analyzed phalloidin-stained cells to detect invadopodia. Confocal microscopy revealed the presence of isolated invadopodia, mostly located in the perinuclear region of HPAF-Ⅱ 3D-spheroids grown without BME. Interestingly, invadopodia seemed more evident in the 3D-spheroids grown in BME (Figure 9C).

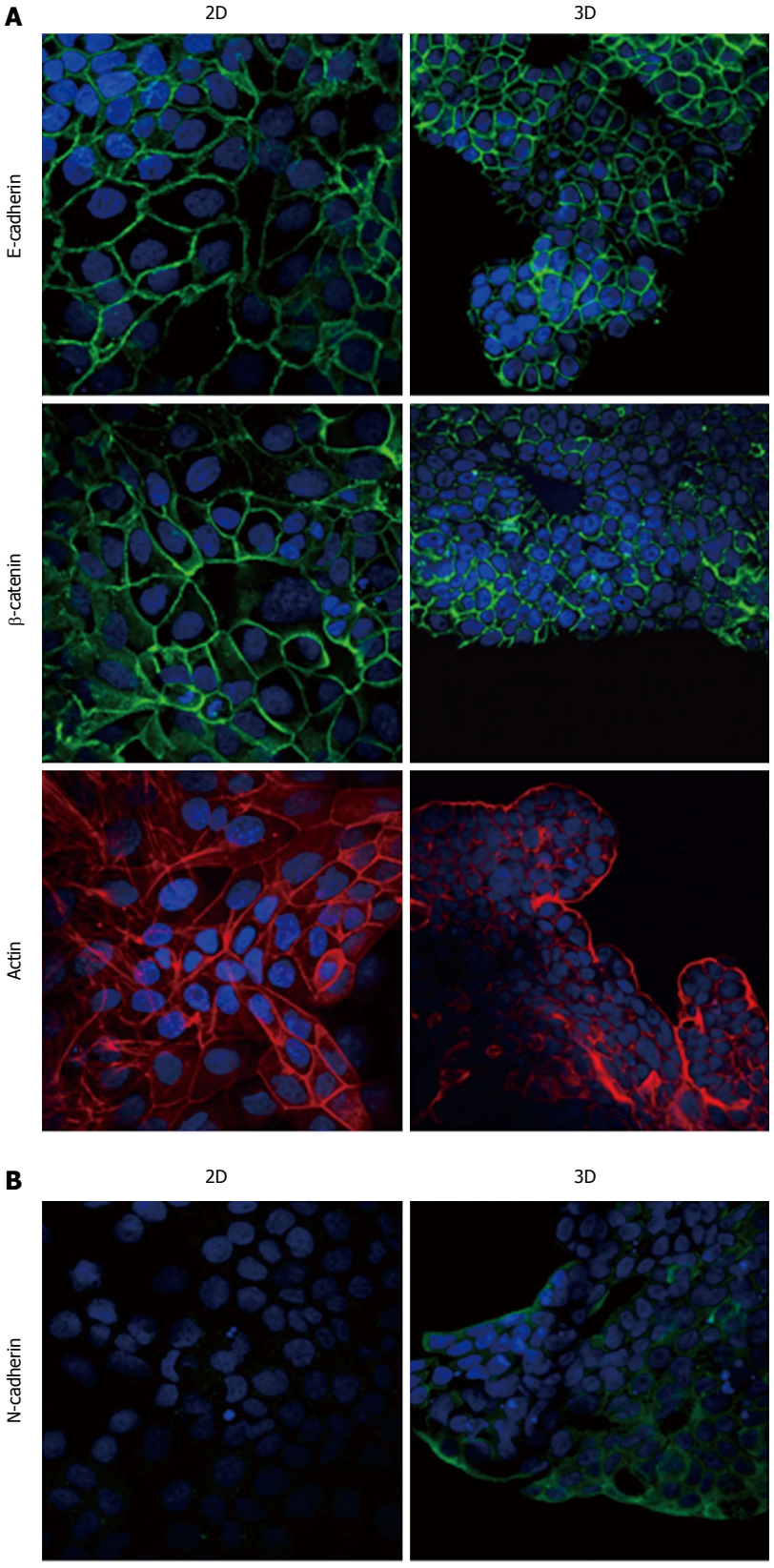
DISCUSSION

EMT is a complex step-wise process characterized by the loss of epithelial adhesion properties following changes in E-cadherin expression patterns, adherens junction disruption, induction of MMPs leading to the disruption of basement membranes, and enhanced migration and invasion[4]. Loss of intercellular adhesion and increased motility promote tumor cell invasion, allowing tumor cells to acquire the capacity to infiltrate surrounding tissue and metastasize at distant sites[22].

An early essential event of EMT is the loss of epithelial phenotype and cell-cell adhesion driven by the down-regulation of E-cadherin, a well-characterized adhesive junction protein expressed in differentiated and polarized epithelial cells. The graded loss of E-cadherin correlates with the aggressiveness of numerous carcinomas and a worsening prognosis, whereas the forced expression of E-cadherin suppresses tumor development in various *in vitro* and *in vivo* experimental tumor models[23]. E-cadherin down-regulation leads to the release of the E-cadherin/β-catenin complex from the plasma membrane, the disruption of cell-cell junctions[24], and to β-catenin nuclear translocation, where it may function as a transcriptional co-activator[25].

It has also been shown that pancreatic cancers display a reduced expression of E-cadherin and an increased expression of N-cadherin which, in primary tumors, are significantly related to histological grade[26] and the invasive/undifferentiated phenotype[27]. However, in 6 out of 7 PDAC commercial cell lines, the expression of E-cadherin was maintained in the cell membrane[13], increasing the relevance of studies aimed at characterizing the phenotype of PDAC cells in relation to the expression of EMT markers, in order to define the role of EMT in PDAC development and progression.

Our immunofluorescence analysis data show that HPAF-Ⅱ, HPAC, and PL45 cells grown in 2D-monolayers and 3D-spheroids are characterized by strong E-cadherin and β-catenin immunoreactivity at the cell-cell boundary, suggesting that adherent junctions are retained by PDAC cells under both experimental conditions. Gene expression analysis showed that


A
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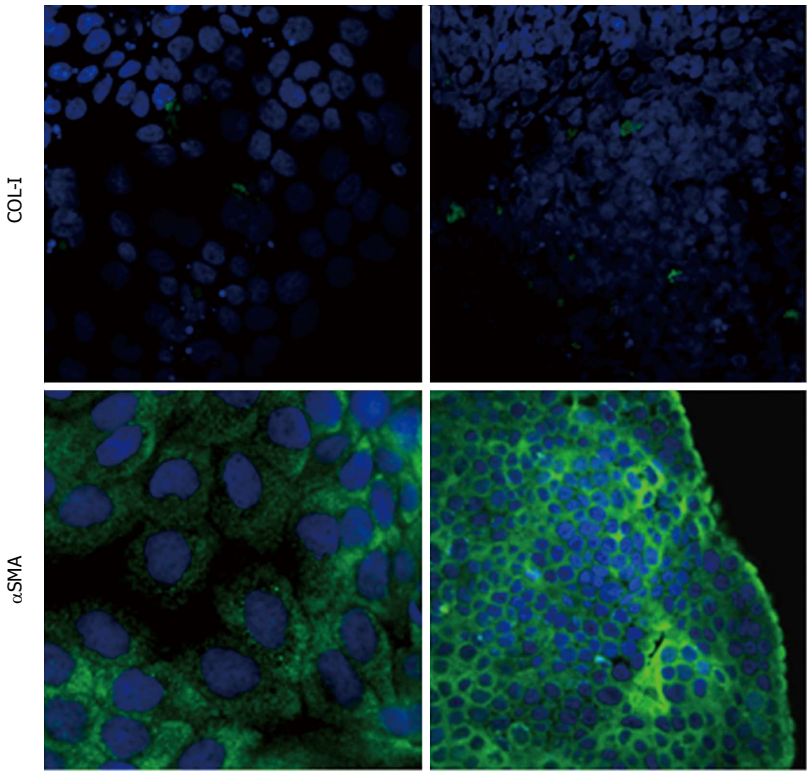

Figure 7 Expression of epithelial-to-mesenchymal transition-related markers in PL45 cells. Micrographs using a confocal microscope showing epithelial (A) and mesenchymal markers (B) in PL45 cells grown in 2D-monolayers and 3D-spheroids. Original magnification: 60 ×.

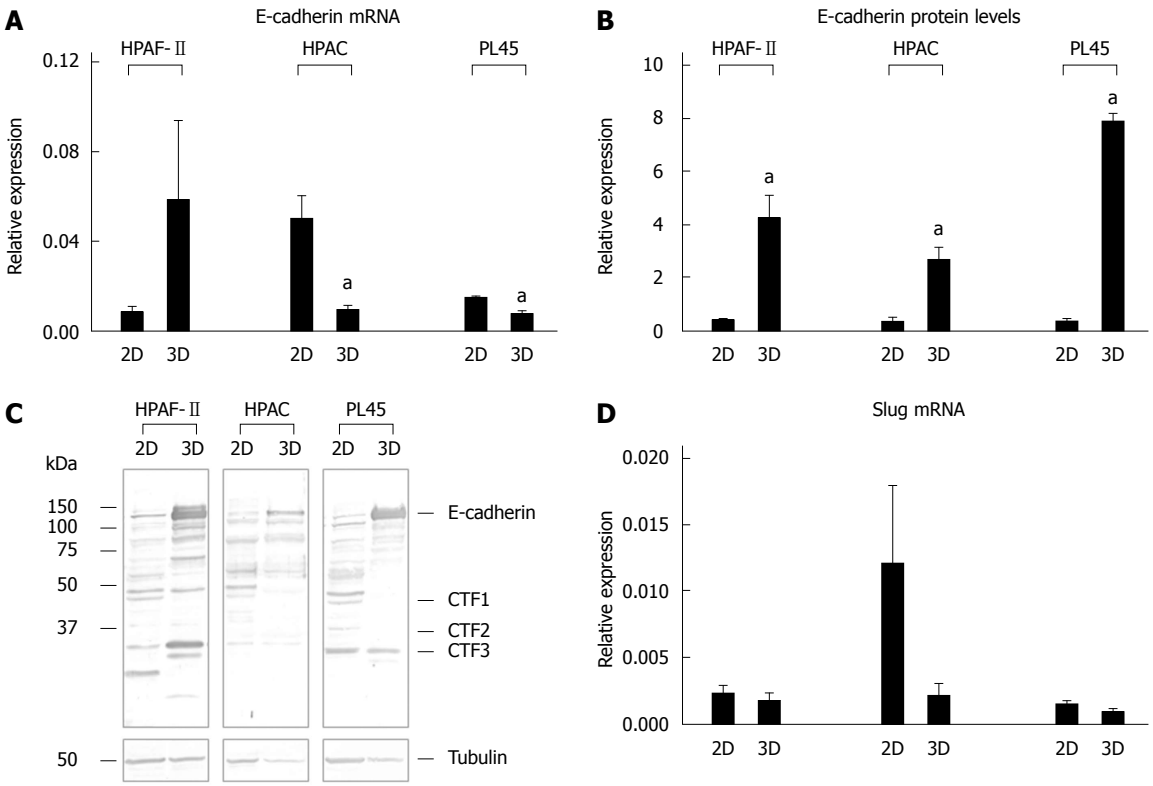

Figure 8 E-cadherin gene and protein expression, and Slug mRNA levels. Bar graphs showing E-cadherin gene (A) and protein expression (B) analyzed by real-time PCR and Western blot, respectively. Data are mean ± SD of duplicate samples run in duplicate experiments; C: Representative Western blot showing the electrophoretic pattern of E-cadherin in lysates obtained from HPAF-Ⅱ, HPAC, and PL45 cells grown in 2D-monolayers or 3D-spheroids; D: mRNA levels for Slug in PDAC cells assayed by real time PCR. Data are mean ± SD of duplicate samples run in duplicate experiments. a: Cohen's d > 2.

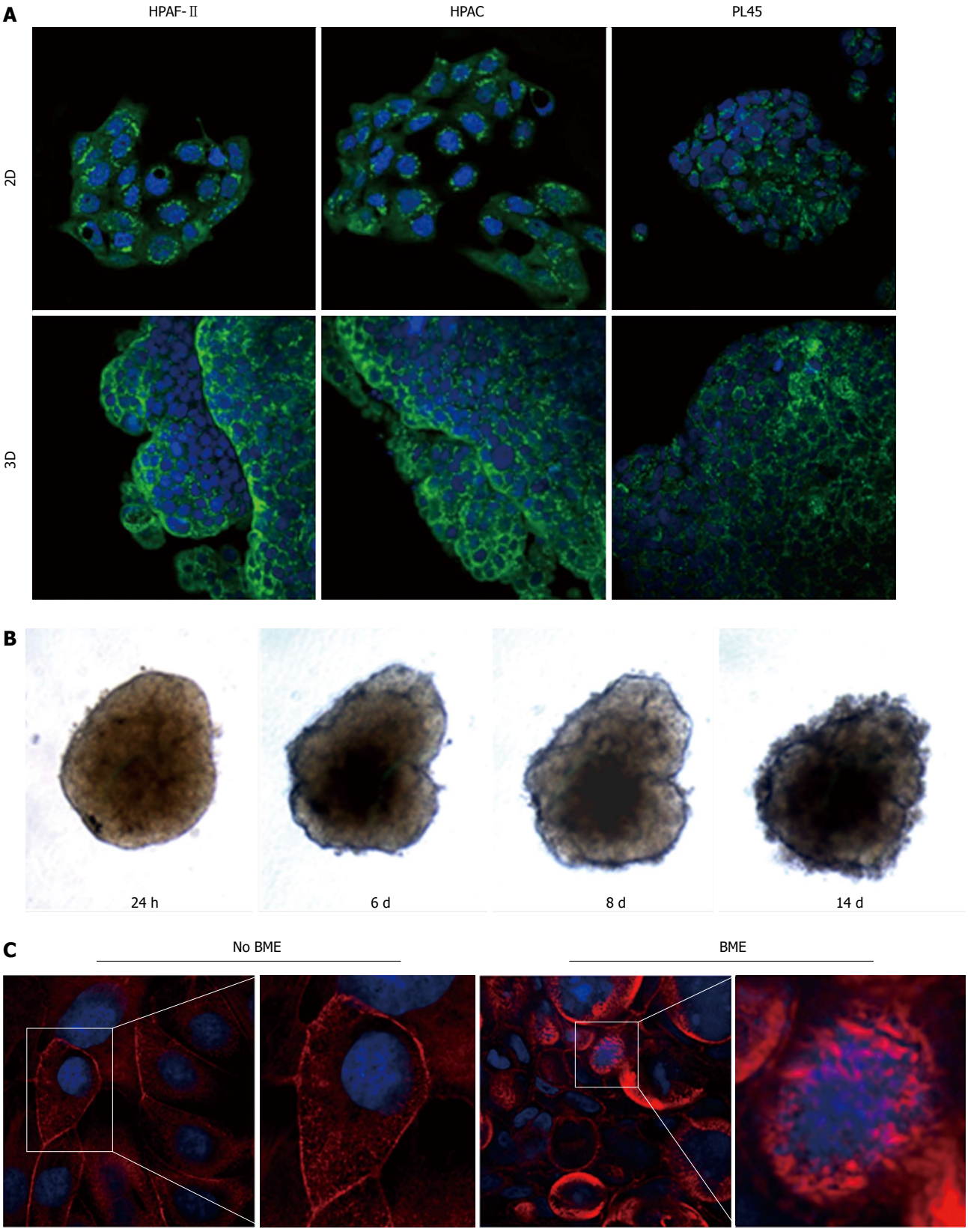

Figure 9 Podoplanin expression and actin cytoskeleton. A: Micrographs with a confocal microscope of PDAC cells grown in 2D-monolayers and 3D-spheroids, showing the expression of podoplanin. Punctuate immunoreactivity is located in the cytoplasm. Original magnification: 60 ×; B: HPAF-Ⅱ 3D-spheroid grown in basement membrane extract (BME) monitored at different time points; C: HPAF-Ⅱ 3D-spheroids grown in the absence or presence of BME were stained using rhodamine-phalloidin to detect actin filaments. Invadopodia are evident boxed. Original magnification: 60 ×.

E-cadherin mRNA levels tended to increase in HPAF-Ⅱ cells grown in 3D-spheroids, but were decreased in HPAC and PL45 spheroids compared to 2D-monolayers. Conversely, E-cadherin was upregulated in PDAC 3D-spheroids at the protein level, suggesting that PDAC cells grown in 3D-spheroids have stronger cell adhesion. The electrophoretic pattern revealed cleavage fragments of E-cadherin in PDAC grown in monolayers, suggesting that the protein underwent partial digestion and was therefore less effective in providing strong cell-cell adhesion under these conditions. However, full length E-cadherin was detected in 3D-spheroids, supporting a stronger cohesion which likely allows and favors collective migration[28]. The presence of E-cadherin degradation fragments may explain the apparent discrepancy between E-cadherin mRNA and protein levels, since they can be generated by post-translational modifications.

The epithelial differentiated phenotype of HPAF-Ⅱ, HPAC, and PL45 was strongly confirmed, especially in 3D-spheroids, using a transmission electron microscope. In fact, PDAC cells grown in 3D-spheroids maintained the junctional complexes and cell polarity typical of columnar simple epithelium lining pancreatic secretory ducts, and exhibited many microvilli on the apical surface. This finding is not in conflict with the behavior of these cells; as previously demonstrated, PDAC cells, even if characterized by a well-differentiated phenotype, are highly malignant and invasive[29].

Our results point to new and important information in understanding the phenotype of these cancer cells in relation to the expression of mesenchymal markers. We showed that PDAC cells in 3D-spheroids retain the expression of the E-cadherin/β-catenin complex at cell boundaries, while N-cadherin is occasionally expressed at the plasma membrane. This finding suggests not only that PDAC cells in 3D-spheroids have undergone the "cadherin switch" typical for EMT, but also that E-cadherin-mediated cell adhesion is retained and probably strengthened by functional adherens cell junctions containing N-cadherin, especially in HPAC cells.

Confocal microscopy confirmed that HPAF-Ⅱ, HPAC, and PL45 exhibit a differentiated epithelial phenotype, as previously suggested[30,31], but some differences in the expression of EMT markers were detected. In particular, mesenchymal markers seemed more evident in HPAC and PL45 cells, with PL45 being the least differentiated. This differing profile could be responsible for the different behavior, but a relationship between differentiation grade, cell migration, and invasion potential has not yet been defined[32].

The concomitant expression of mesenchymal markers, such as αSMA, in both 2D-monolayers and 3D-spheroids supports the hypothesis that PDAC cells underwent EMT. The expression of EMT markers such as Twist, Snail, Zeb1, and Zeb2 was almost undetectable, in line with the high expression of E-cadherin and the maintenance of adherens junctions. Slug was detected in PDAC cells at low levels, particularly in HPAC cells, in both 2D-monolayers and 3D-spheroids. An inverse correlation between Snail and E-cadherin at the mRNA level in pancreatic cancer cells was previously reported, while Slug expression in pancreatic cancer was reported to have no evident relationship with decreased expression of E-cadherin[7]. To explain this apparent inconsistency, it was suggested that the transient expression of Snail might be involved in inducing the invasion process, whereas Slug might be involved in the maintenance of the migratory invasive phenotype[7].

Some cells in 3D-spheroids expressed COL-I, a protein typical of mesenchymal-like cells. We feel this is an interesting finding that demonstrates the relevance of the 3D arrangement in determining cell phenotype and, therefore, supports the importance of 3D experimental models in cancer research.

E-cadherin expression and functional adherens junctions seem more evident in 3D-spheroids, suggesting that higher cell adhesion could favor collective cell migration in PDAC cells by linking them together and ensuring tissue integrity during collective cell migration. The hypothesis of PDAC cells moving cohesively in a collective migration is also supported by the observation that no single invading cells were previously detected in 3D reconstructed models, indicating that single cell invasion does not occur in PDAC. Moreover, increased E-cadherin was shown in the leading edge of migrating epithelial sheets[33], supporting collective migration for these cancer cells. In contrast with individually migrating cells, during collective migration the rear of the front cell retains intact cell-cell junctions to the successor cell, thereby mechanically holding the cells together and augmenting the efficiency of paracrine cell-cell signaling and multicellular coordination[34,35].

Podoplanin is a small mucin-like protein up-regulated in a number of different cancers, suggesting a role in tumor progression[36-39]. Although the physiological function of podoplanin is still unknown and its functional contribution to tumor progression has remained elusive, podoplanin expression was observed in cells invaded by collective migration[40]. Results from immunofluorescence analysis show podoplanin expression in HPAF-Ⅱ, HPAC, and PL45, according to the hypothesis of collective invasion of PDAC cells. This is consistent with the evidence of small clusters of cells detaching from spheroid surfaces observed at TEM, and with the results of the behavior of HPAF-Ⅱ spheroids in BME, showing that spindle-like projections extending in the BME were not evident, but small groups of cells did detach from the spheroid surface to invade the surrounding matrix. Furthermore, we detected frequent invadopodia, known as specialized podosomes, which release matrix metalloproteinases and characterize collectively invading cancer cells[41]. These invadopodia were more evident in the presence

of BME, as well as being evident under TEM.

Considered as a whole, our data could contribute to clarifying the role of EMT in PDAC progression, provide additional correlative evidence that PDAC cells express EMT markers, and point to relevant differences in the phenotype of PDAC cells grown in 3D-spheroids. In fact, under these experimental conditions, PDAC cells are characterized by functional adherens junctions and concomitant expression of mesenchymal markers, such as N-cadherin and COL-I, which are almost undetectable in PDAC cells grown in 2D-monolayers.

Our results support the use of 3D cultures in biomedical research to bridge the gap between traditional cell cultures and *in vivo* settings in order to more clearly understand the biology of PDAC[14-17,42]. Since 3D cultures seem to provide excellent information on PDAC cell phenotype[43,44], they could represent a pre-clinical model for identifying and validating tumor markers, as well as allowing for the study of new therapeutic tools for PDAC.

ACKNOWLEDGMENTS

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COMMENTS

Background

The functions of living tissues can be mimicked in three-dimensional (3D) cell cultures, thereby providing a method of decoding the information encoded in the tissue architecture. The authors analyzed the effect of 3D-arrangement on the expression of some key markers of epithelial-to-mesenchymal transition (EMT) in pancreatic adenocarcinoma (PDAC) cells cultured in either 2D-monolayers or 3D-spheroids.

Research frontiers

Although 3D-cell cultures represent a well-established experimental condition to study the effect of 3D-arrangement on cell phenotype in different cell types, few studies have been performed using 3D-cell cultures of PDAC cells. Some discrepancies were described in the expression of E-cadherin in PDAC commercial cell lines and tissue fragments, increasing the relevance of studies aimed at characterizing the phenotype of PDAC cells in relation to the expression of EMT markers.

Innovations and breakthroughs

The overall information provided by this study supports the use of 3D-cultures in biomedical research to bridge the gap between traditional cell cultures and *in vivo* settings. This approach places cultured cells in an environment that more closely represents and mimics the complex 3D structure of living tissues, in order to more clearly understand the biology of PDAC. The results show that a 3D-cell culture model could provide deeper insight into understanding the biology of PDAC, thereby allowing for the detection of important differences in the phenotype of PDAC cells grown in 3D-spheroids. This study contributes to the clarification EMT's role in PDAC progression, and provides additional correlative evidence of EMT marker expression in PDAC cells.

Applications

3D cultures offer a potential pre-clinical model for identifying and validating tumor markers, as well as allowing for the study of new molecular tools to inhibit signaling pathways and target EMT transcription factors.

Peer-review

This manuscript describes EMT phenomena in 3D-cell cultures using three kinds of pancreatic cancer cell lines. The authors investigated ultrastructural characterization of EMT with transmission electron microscopy and expression of EMT-associated proteins, such as αSMA and E-cadherin. A marked EMT phenomenon was observed in 3D-cell cultures compared to 2D cultures. The results are very interesting.

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ORIGINAL ARTICLE

Basic Study

Role of estrogen receptor β selective agonist in ameliorating portal hypertension in rats with CCl_4-induced liver cirrhosis

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Abstract

AIM: To investigate the role of diarylpropionitrile (DPN), a selective agonist of estrogen receptor β (ERβ), in liver cirrhosis with portal hypertension (PHT) and isolated hepatic stellate cells (HSCs).

METHODS: Female Sprague-Dawley rats were ovariectomized (OVX), and liver cirrhosis with PHT was induced by CCl_4 injection. DPN and PHTPP, the selective ERβ agonist and antagonist, were used as drug interventions. Liver fibrosis was assessed by hematoxylin and eosin (HE) and Masson's trichrome staining and by analyzing smooth muscle actin expression. Hemodynamic parameters were determined *in vivo* using colored microspheres technique. Protein expression and phosphorylation were determined by immunohistochemical staining and Western blot analysis. Messenger RNA levels were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). Collagen

gel contraction assay was performed using gel lattices containing HSCs treated with DPN, PHTPP, or Y-27632 prior to ET-1 addition.

RESULTS: Treatment with DPN *in vivo* greatly lowered portal pressure and improved hemodynamic parameters without affecting mean arterial pressure, which was associated with the attenuation of liver fibrosis and intrahepatic vascular resistance (IHVR). In CCl_4-treated rat livers, DPN significantly decreased the expression of RhoA and ROCK Ⅱ, and even suppressed ROCK Ⅱ activity. Moreover, DPN remarkedly increased the levels of endothelial nitric oxide synthase (eNOS) and phosphorylated eNOS, and promoted the activities of protein kinase G (PKG), which is an NO effector in the liver. Furthermore, DPN reduced the contractility of activated HSCs in the 3-dimensional stress-relaxed collagen lattices, and decreased the ROCK Ⅱ activity in activated HSCs. Finally, *in vivo*/*in vitro* experiments demonstrated that MLC activity was inhibited by DPN.

CONCLUSION: For OVX rats with liver cirrhosis, DPN suppressed liver RhoA/ROCK signal, facilitated NO/PKG pathways, and decreased IHVR, giving rise to reduced portal pressure. Therefore, DPN represents a relevant treatment choice against PHT in cirrhotic patients, especially postmenopausal women.

Key words: Portal hypertension; Estrogen receptor; Rho-kinase signaling; Nitric oxide; Hepatic stellate cells

Core tip: Liver cirrhosis and portal hypertension (PHT) are subject to gender and estrogen levels. The aim of the present study was to investigate whether estrogen receptor β selective agonists could ameliorate intrahepatic resistance and mitigate PHT in rats with CCl_4-induced cirrhosis, and uncover the underlying mechanism by investigating RhoA/ROCK and NO/PKG signaling. The authors propose that treatment with an estrogen receptor β selective agonist could improve cirrhotic PHT *via* regulating RhoA/ROCK and NO/PKG signaling.

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INTRODUCTION

Increased intrahepatic vascular resistance (IHVR) to portal blood flow is a major contribution to portal hypertension (PHT) in liver cirrhosis[1,2], and decreased splanchnic vascular resistance worsens and maintains the increased portal pressure (PP)[2,3]. Over the past 20 years, with a keen grasp on hepatic microcirculation, a dynamic component involving changes in hepatic vascular tone has been demonstrated to contribute to IHVR; hence, increased vascular tone augments IHVR[4,5]. Apart from structural changes (fibrosis, vascular remodeling, vascular occlusion, and nodule formation), activated hepatic stellate cells (HSCs), contraction of intrahepatic vascular smooth muscle cells (VSMCs) and decreased levels of NO vasodilator, all play a critical role in contributing to increased IHVR[4,6]. It is well known that the intrahepatic upregulation of RhoA/ROCK signaling, as well as the inhibition of NO/PKG signaling, contributes to increased IHVR[7-9]. Furthermore, the two pathways regulate each other, maintaining the balance between phosphorylation and dephosphorylation of myosin light chains (MLC)[9-11]. Thus the two pathways are crucial therapeutic targets to inhibit the increased IHVR and PP occurring in cirrhosis.

Epidemiological studies have reported the male to female ratio among patients with cirrhosis is in the range of 2.3:1-2.6:1; moreover, menopause increases the susceptibility to cirrhosis and PHT[12,13]. Animal experiments and clinical trials have provided consistent evidence for the protective effect of endogenous and exogenous estrogen on liver fibrosis[12-16]. However, the administration of exogenous estrogen had its potential risks, causing their clinical use[17] to be impeded. Fortunately, previous studies regarding estrogen receptor (ER) subtypes have indicated that estrogen receptor β (ERβ) gives rise to few side effects of estrogen[18], while ERα mediates the majority effects of estrogen on classic estrogen target tissues, as well as their associated side effects[19]. Interestingly, high ERβ expression levels and low ERα expression levels were observed in both men's and women's normal and fibrotic livers, and HSCs had functional ERβ, rather than ERα, which responded directly to estradiol (E2) exposure[20]. ERβ selective agonists hold the key to producing protective effects of estrogens on liver cirrhosis and PHT, while reducing undesired side effects[21].

Therefore, this study investigated the effect of diarylpropionitrile (DPN), an ERβ selective agonist, on the intrahepatic RhoA/ROCK and NO/PKG pathways, and on hepatic hemodynamics systemically as well.

MATERIALS AND METHODS

Animals

Female SD rats - initially weighing 180-200 g - were acquired from the Laboratory Animal Center of School of Medicine, Shanghai Jiao Tong University, China. Under the constant temperature of 21 ℃, rats were exposed to a light/darkness cycle of 12 h/12 h, and accessed to water and standard rat chow. All animal

experiments conformed to guidelines on caring and using lab animals which were reviewed by the Research Ethics Committee of Renji Hospital (No. RJ-20151211).

Treatment regimens

Rats were assigned to a sham-operated control group (n = 15) or an ovariectomized (OVX) group (n = 45) in a random way. The rats were intraperitoneally injected with ketamine (100 mg/kg per body weight) and xylazine (12 mg/kg per body weight) for anesthesia. The surgical procedure was performed from a midline back incision and both ovaries were removed. The control group received the same incisions and the two ovaries were explored but not excised. The animals were allowed 2 wk for recovery. OVX rats were divided into three groups with 15 in each, as below: OVX + CCl_4 group, OVX + CCl_4 + DPN group and OVX + CCl_4 + DPN + PHTPP group.

CCl_4 administration

The rats needed to weigh and administer mixed food on a daily basis. For the OVX + CCl_4 group, the subcutaneous injection at a dose of 4 mL/kg was conducted twice a week while doubling dosage for the first injection, as 400 mL/L CCl_4 with olive oil needs to be done. After 14-16 mo, this procedure led to micro nodular cirrhosis with PHT. In addition to this, the OVX + CCl_4 + DPN group was treated subcutaneously with 30 nmol/100 g DPN in 1 mL dimethyl sulfoxide (DMSO), twice weekly. Along with CCl_4 and DPN, the OVX + CCl_4 + DPN + PHTPP group also received 30 nmol/100 g PHTPP in 1 mL DMSO, twice weekly. The control group was injected with 1 mL DMSO, twice weekly. After 14 to 16 mo, CCl_4 and drug injections were stopped within 6 d prior to the start of experiments. Although there were 15 rats in each group at the beginning of the study, the number of the rats decreased to 11, 13 and 12 in the OVX + CCl_4, OVX + DPN + CCl_4 and OVX + DPN + PHTPP + CCl_4 groups, respectively, due to death caused by illness. Five rats from each group were sacrificed for tissue harvesting. Sample livers were kept in formaldehyde or snap-frozen by liquid nitrogen under the temperature of -80 ℃. The mesenteric arteries were used to detect mesenteric arteriole reactivity. The other rats were used for hemodynamic studies and their blood was used to analyze biochemical parameters.

Hemodynamic studies

When rats were given ketamine anesthesia (100 mg/kg, imp), in a median laparotomy, a PE-50 catheter was inserted into a small ileocaecal vein and guided to the portal vein to measure PP. A PE-50 catheter was introduced to a left femoral artery to measure mean arterial pressure (MAP). An additional PE-50 catheter was inserted from a right carotid artery leading to the left ventricle, which was used for microsphere injection. The femoral artery catheters and the portal vein were in connection with a pressure transducer (M100613, United States Philips Corporation). The PP and MAP were recorded on a multi-channel recorder (COLIN, BP508, Japan). The zero reference point referred to the spot of 1 cm above the operating table.

The Dye-Trak microsphere technique was performed as per previous description. Briefly, the 1-min withdrawal (0.65 mL/min) of a reference sample was conducted with a continuous extraction pump (ALC-IP900, shanghai, china). Suspending in the solution of 0.3 mL saline with 0.05% Tween, approximately 300000 yellow microspheres of 15.5 μm in diameter (Triton Technologies, San Diego, California, United States) were injected into the left ventricle within 10 s of starting blood withdrawal. Suspending in a solution as same as yellow ones, 150000 blue microspheres was injected into an ileocaecal vein within 30 s to evaluate mesenteric portal-systemic shunt volume. Ten minutes later, the rats were sacrificed by injecting KCl intravenously. The blood and tissue samples were assimilated by a portion of 3.8 mL of 5.3 mol/L KOH and 0.5 mL Tween 80, and were subsequently boiled for 1 h. Then ready samples were processed by vortex and filtered with Whatman Nucleopore filters (Whatman International, Maidstone, United Kingdom). Colors were extracted from the filtered microspheres by using 0.2 mL dimethyl formamide and measured by absorption spectrophotometry. Hemodynamic parameters were measured and calculated according to standard methods[22,23]. Afterwards, with the software of Triton Technologies, cardiac output and organ blood flow were calculated and expressed based on 100 g per body weight. Splanchnic perfusion pressure was obtained by deducting PP from MAP. Splanchnic vascular resistance was rated by the splanchnic perfusion pressure to the splanchnic blood flow. Mesenteric portal-systemic shunt flow was derived from the fraction in the lung out of total injected blue microspheres. Hepatic portal-vascular resistance was estimated as PP divided by the sum of gastrointestinal and splenic perfusion minus mesenteric portal-systemic shunt flow. Systemic vascular resistance (SVR) was defined as the ratio of MAP to cardiac output.

Histological and immunohistochemical assessment

HE, immunohistochemical, and trichrome collagen staining were applied to examine liver sections (4 mm) on glass slides with silane coating. Liver sections were evaluated randomly by an accomplished liver pathologist yet unfamiliar with animal groups.

For immunohistochemistry, primary antibodies (Cell Signaling Technology, Danvers, MA) were used at a dilution of 1:200 (phosphor-Thr18/Ser19-PML) or 1:100 (α-SMA) to incubate liver sections, after the incubation in the streptavidin-peroxidase complex. Peroxidase conjugates were then viewed in diaminobenzidine (DAB) solution. Afterwards, the prepared livers were

processed by hematoxylin counterstaining and then covered with a plate.

Collagen contents were quantified using Masson's trichrome collagen stain, and the Masson-stained areas are reported to be its ratio to the total area. The positive areas were analyzed using Image J software. The liver sections were then averagely valued among five rats from each group.

Quantitative RT-PCR

An array of processes need to be done, including separating RNA from 30 mg shock-frozen hepatic tissue with TRIzol (Invitrogen), using MMLV reverse transcriptase (Invitrogen) to perform reverse transcription, conducting quantitative RT-PCR (qRT-PCR) with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), and preparing primers and probes for RT-PCR with Primer Express Software (Applied Biosystems, Foster City, CA). 18S rRNA was used for the endogenous control. The used primer sequences were the following: GAPDH, (Forward) GGAGTCCACTGGCGTCTTC and (Reverse) GGCATTGCTGATGATCTTGAGG; RhoA, (Forward) GGCAGAGATATGGCAAACAGG and (Reverse) TCCGTCTTTGGTCTTTGCTGA; ROCK-Ⅱ, (Forward) CCCGATCATCCCCTAGAACC and (Reverse) TTGGAGCAAGCTGTCGACTG.

Western blot and antibodies

Shock-frozen section samples were processed by homogenization in buffer containing 25 mmol/L Tris/HCl, 5 mmol/L ethylenediamine tetraacetic acid, 10 μmol/L phenylmethanesulfonyl fluoride, 1 mmol/L benzamidine, and 10 μg/mL leupeptin. Liver samples were put in the buffer for dilution. Homogenate protein concentrations were determined using the BCA Protein Assay kit (Beyotime, Haimen, China). Samples (40 μg of protein/lane) were assayed by SDS-PAGE (15% gels for RhoA and p-MLC; 8% for ROCK, iNOS, eNOS, and p-eNOS; and 10% for moesin, p-moesin, VASP, p-VASP, and α-SMA). After electrophoresis, protein was shifted under the effect of a 250 mA current for 1.5 h to a polyvinylidene difluoride membrane which was stemmed by 5% BSA for 2 h and treated as below in primary antibodies at the temperature of 4 ℃ overnight: DAPDH, ERβ, α-SMA, iNOS, eNOS, and p-eNOS (Ser^{1177}) from Abcam (Cambridge, United Kingdom); RhoA, ROCKII, moesin, p-moesin (Thr^{558}), VASP, p-VASP (Ser^{239}), MLC, and p-MLC (Thr^{18}/Ser^{19}) from Santa Cruz Biotechnology (Santa Cruz, CA). The membrane was then processed for 1 h with appropriate secondary antibodies (Abcam) at a 1:5000 dilution. Fluorescent signals were detected with an Odyssey Imaging System (Li-Cor Biosciences, Lincoln, NE). GAPDH served as an internal control.

Assessment of PKG, ROCK and MLC activities

PKG activity was assessed and indicated by the phosphorylation level of endogenous substrate VASP at $Ser239$[8,22]. ROCK activity was evaluated by measuring the phosphorylation of endogenous substrate, moesin, at Thr^{558}[8,22]. MLC activity was assessed by measuring the phosphorylation of MLC at Thr^{18}/Ser^{19}[24]. The analysis was performed by Western blot with site- and phosphor-specific antibodies.

Cell culture

HSCs were separated from male SD rats with the weights of 300-400 g as per previous description[24]. Technically, after in situ sequential perfusion of the solutions of collagenase IV (Sigma, St. Louis, United States) and pronase E (Merck, Darmstadt, Germany) to hepatic samples, decentralized cells were separated by density gradient centrifugation with Optiprep (Nycomed, Sweden). Cells were obtained at a density less than 1.053 (9% Optiprep). Viability and purity were determined to be higher than 95% as per Trypan blue exclusion and morphological characteristics. Then cells were plated onto uncovered plastic culture plates, being cultivated with Dulbecco's Modified Eagle Medium without Phenol Red (DMEM; Invitrogen), which was complemented with 10% fetal bovine serum, 0.6 IU/mL insulin, 2 mmol/L glutamine, and 1% antibiotic-antimycotic solution (Invitrogen), and was renewed every 48-72 h.

Collagen gel contraction assay

Collagen gel contraction experiments were conducted upon slight modifications[25] as per previous descriptions. Briefly, hydrated collagen gels were produced with rat tail tendon collagen I (Becton Dickinson Labware, Bedford, MA) and made adjustments with 0.1 N NaOH and 10 × PBS, to a final collagen concentration of 1.2 mg/mL and pH 7.4 at 4 ℃. A 500 μL portion of collagen solution was put into wells of a 24-well tissue culture dish for 1 h incubation at 37 ℃. Then HSCs were layered on top of the collagen lattice of 5×10^5 cells/mL. Twenty-four hours after adding 1 mL/well of serum free culture medium and the starvation process, lattices in stability were flushed twice with 1 × PBS. After pretreatment with DPN (10^{-7} mol/L), DPN (10^{-7} mol/L) + PHTPP (10^{-7} mol/L), or Y-27632 (10^{-5} mol/L) for 30 min, HSCs were exposed to ET-1 (10^{-8} mol/L, Roche Diagnostics, Brussels, Belgium). Buffer without ET-1 was used as a control. Gels were immediately detached with the tip of a 100 μL pipette from the plates in the pattern of gentle circumferential dislodgment. Digital photos were obtained to monitor the change in lattice area 4 h after addition of the contractile agonist. All information was drawn from studies of more than three sets of triple collagen lattices by cultured HSCs out of three different rat HSC separations.

Analysis of Rho kinase and MLC activity in HSCs

Activated HSCs grown on culture dishes were starved for 24 h. HSCs were pretreated with DPN (10^{-7} mol/L), DPN (10^{-7} mol/L) + PHTPP (10^{-7} mol/L), or Y-27632

Table 1 Biochemical parameters of the different treatment groups

Group	ALT (U/L)	AST (U/L)	Bilirubin (mg/dL)	Albumin (g/L)	BUN (mmol/L)	Scr (μmol/L)
Control (n = 8)	43.3 ± 7.7	124 ± 14	0.3 ± 0.1	34.1 ± 3.4	8.7 ± 1.2	22.4 ± 4.0
OVX + CCl_4 (n = 6)	166 ± 10.1[a,b]	439 ± 19[a,b]	3.4 ± 0.3[a,b]	21.5 ± 2.8[a,b]	16.7 ± 1.8[a,b]	35.0 ± 4.7[a,b]
OVX + CCl_4 + DPN (n = 7)	86.1 ± 8.7[a]	211 ± 15[a]	1.7 ± 0.2[a]	31.0 ± 3.3	9.6 ± 1.5	24.9 ± 4.5
OVX + CCl_4 + DPN + PHTPP (n = 6)	168 ± 10.2[a,b]	436 ± 23[a,b]	3.3 ± 0.4[a,b]	20.8 ± 2.0[a,b]	16.5 ± 2.1[a,b]	37.5 ± 4.6[a,b]

[a]P < 0.05 *vs* control group; [b]P < 0.05 *vs* DPN group. OVX: Ovariectomized; SVR: Systemic vascular resistance; DPN: Diarylpropionitrile.

(10^{-5} mol/L) for 30 min, prior to exposure to ET-1 (10^{-8} mol/L) for 4 h. Buffer without ET-1 was used as a control. ROCK and MLC activities were assessed by monitoring the phosphorylation levels of moesin at Thr^{558} and MLC at Thr^{18}/Ser^{19}, respectively[22,26].

Statistical analysis

Data are expressed as mean ± SE. After the Bonferroni/Dunn or Mann-Whitney *U* test, ANOVA was applied to compare among groups (SPSS 21 for Windows, SPSS Inc., Chicago, IL). A *P*-value < 0.05 had great statistical significance. With regards to analyzing concentration response curves, the information was seated by nonlinear regression in the Prism computing project (Graph Pad Software Inc., San Diego, CA).

RESULTS

Biochemical parameters

CCl_4 caused a great increase of the analyzed biochemical parameters, including ALT, AST and bilirubin, and a significant decrease in albumin. However, treatment with DPN decreased the ALT, AST and bilirubin levels, and increased the albumin levels in PHT rats. PHTPP counteracted the effect of DPN (Table 1).

Morphological characteristics of the rat livers

CCl_4 caused significant hepatocyte steatosis, fibrous proliferation of interlobular portal areas, and formation of pseudolobules and tubercles, whereas DPN attenuated these phenomena (Figure 1A). This could be verified by trichrome staining (Figure 1B) from a histological perspective. Collagen content quantification demonstrated that the collagen volume fraction increased in CCl_4-induced cirrhotic rats (24.8% ± 4.8%) in comparison with the control group (2.1% ± 0.5%), however, DPN treatment played a significant role in inhibiting the secretion of collagen (17.0% ± 4.0%). There was no statistically significant difference in collagen content between the PHTPP group (22.9% ± 4.9%) and the CCl_4-induced group (Figure 1C).

Expression of α-SMA in rat livers

The expression of α-SMA correlated with the activity of HSCs and the degree of cirrhosis. Immunohistochemical staining and Western blot analysis against α-SMA consistently showed a significant increase in α-SMA expression in the OVX + CCl_4 and OVX + CCl_4 + DPN + PHTPP groups, while DPN treatment greatly decreased the expression of hepatic α-SMA in CCl_4-treated rats (Figure 2A-D).

Expression of ERβ in rat livers

ERβ expression was present in the rat livers of all of four groups. The expression of ERβ in DPN treated rats was slightly higher than that in the other three groups, although there was no statistically significant difference between all groups (Figure 3A-B)

In vivo hemodynamic studies

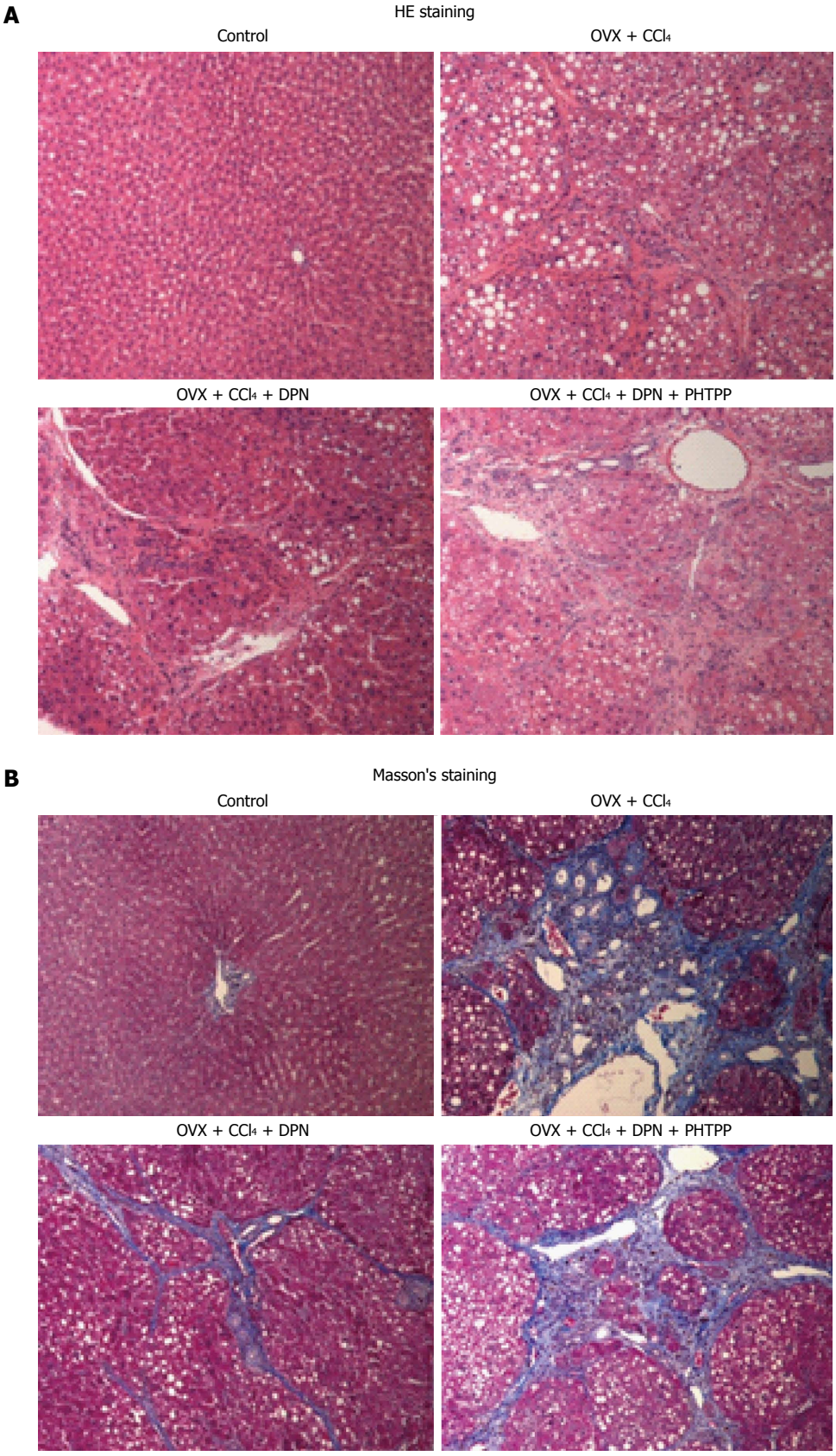
In the OVX + CCl_4 group there were markedly increased PP and IHVR compared to the control group. However, treatment with DPN significantly decreased PP and IHVR, while PHTPP counteracted the effect of DPN (Table 2). With regards to hyperdynamic circulation, the OVX + CCl_4 treated rats presented markedly increased CO and PVI, and decreased MAP, TPR and SVR. Nevertheless, DPN significantly decreased CO and PVI, and increased SVR, but did not affect MAP and TPR. PHTPP counteracted the effect of DPN on CO, PVI and SVR (Table 2).

Effect of DPN on the RhoA/ROCK pathway in rat livers

In comparison with those sham-operated non-cirrhotic rats, both RhoA and ROCK protein levels were dramatically improved in the OVX + CCl_4 rat livers. Treatment with DPN significantly down-regulated RhoA and ROCK protein levels, though they remained higher than those in sham-operated rats. In contrast, treatment with PHTPP counteracted DPN (Figure 4A-B). In all four groups, the mRNA expression levels of both RhoA and ROCK were consistent with RhoA and ROCK protein levels (Figure 4C). As a ROCK activity indicator, the moesin phosphorylation was examined by Western blot. Western blot analysis showed that p-moesin (Thr^{558}) levels were significantly enhanced in OVX + CCl_4 rat livers. DPN treatment significantly decreased the level of p-moesin (Thr^{558}), while PHTPP treatment counteracted the effect of DPN. These differences were not in association with variation of overall moesin levels, alike in all groups (Figure 4D-E).

Effect of DPN on the NO/PKG pathway in rat livers

There was no distinction between the intrahepatic eNOS expression levels of the OVX + CCl_4 group and the control group, but there was an important decrease in


A
HE staining
Control
OVX + CCl4
OVX + CCl4 + DPN
OVX + CCl4 + DPN + PHTPP
B
Masson's staining
Control
OVX + CCl4
OVX + CCl4 + DPN
OVX + CCl4 + DPN + PHTPP

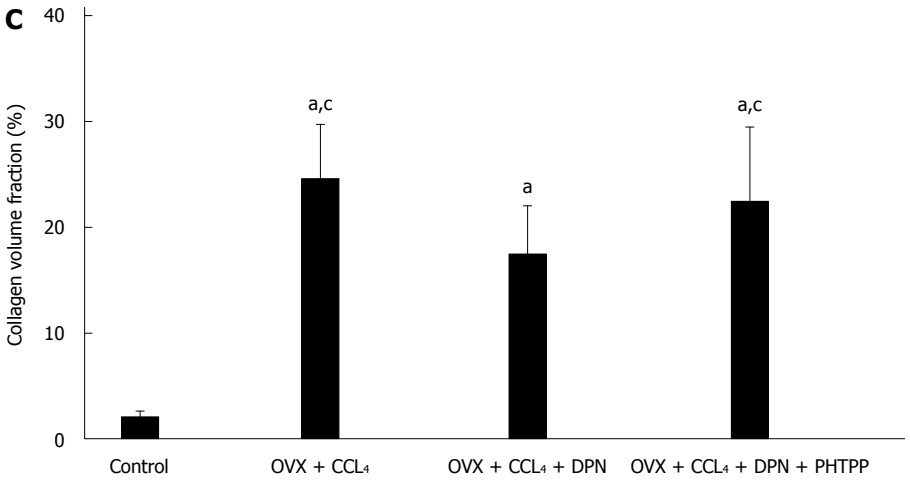


Figure 1 Therapeutic effects of diarylpropionitrile on hepatic fibrosis in CCl_4-treated rats. Histological images of rat livers stained with HE (A) or Masson's staining (B) (magnification × 100) and semi-quantitative measurement of Masson's staining (C). $^{a}P < 0.05$ *vs* control group; $^{c}P < 0.05$ *vs* DPN group. DPN: Diarylpropionitrile.

intrahepatic eNOS phosphorylation amount. Treatment with DPN not only markedly increased the expression of eNOS, but also significantly up-regulated p-eNOS (Ser^{1177}) levels in the cirrhotic livers of OVX rats (Figure 5A-B).

PKG activity was evaluated by measuring the phosphorylation of its endogenous substrate, VASP at Ser^{239}. Western blot analysis using p-VASP (Ser^{239}) antibodies revealed that p-VASP (Ser^{239}) levels remained unchanged in the OVX + CCl_4 and PHTPP treated rats, compared to the control group. However, DPN treatment significantly enhanced intrahepatic p-VASP (Ser^{239}) levels. No differences in total VASP expression were found between treatment groups. Therefore, it can be said that DPN increases PKG activity in the livers of OVX + CCl_4 rats (Figure 5A-B).

Regarding the expression of iNOS, Western blot analysis revealed that the marked increase in iNOS expression seen in the cirrhotic livers OVX rats could be inhibited by DPN (Figure 5A-B).

MLC activity

The RhoA/ROCK and NO/PKG pathways maintain the balance between phosphorylation and dephosphorylation of MLC[9-11]. Thus, we investigated the level of p-MLC (Thr^{18}/Ser^{19}) using Western blot analysis. The results revealed that p-MLC (Thr^{18}/Ser^{19}) levels greatly increased in the OVX + CCl_4 rat livers compared to control rats. However, treatment with DPN significantly decreased the level of p-MLC (Thr^{18}/Ser^{19}), while the addition of PHTPP increased the level of p-MLC (Thr^{18}/Ser^{19}) once more (Figure 6A-B). Therefore, DPN inhibited MLC activity in the OVX + CCl_4 rat livers, which reduced the contraction of intrahepatic VSMCs.

Collagen gel contraction assay

The contraction was assessed using a model in which subcultured cells were grown on top of gel lattices composed of type 1 collagen. The lattice contraction in ET-1 processed cells registered stronger than in normal cells, producing gels by 51.8% ± 7.1% and 82.6% ± 8.9% of the original size, respectively. Pretreatment with 10^{-7} mol/L DPN or 10^{-5} mol/L Y-27632 significantly reduced the contraction of HSCs, reducing the gel areas to only 73.8% ± 8.3% and 77.7% ± 9.6%, respectively. However, pretreatment with both 10^{-7} mol/L DPN and 10^{-7} mol/L PHTPP produced HSC contraction similar to that of the ET-1 control, 54.4% ± 7.2% of the initial gel area (Figure 7A-B). Thus, DPN inhibited the ET-1-induced HSC contraction, and the inhibitory capacity of DPN was similar to that of the ROCK inhibitor Y-27632.

Expression of ERβ in HSCs

Expression of ERβ existed in all of the five cell treatment groups. Furthermore, there were no statistically significant differences in the ERβ expression of the five groups (Figure 7C-D).

Effect of DPN on ROCK activity in HSCs

Western blot analysis showed that ET-1-induced activation significantly increased the level of p-moesin (Thr^{558}) in HSCs compared to the control group. Treatment with DPN reduced the phosphorylation of moesin in HSCs, while addition of PHTPP returned the p-moesin (Thr^{558}) level to that seen in the ET-1 group. Treatment with Y-27632 was more effective than DPN in inhibiting the level of p-moesin (Thr^{558}) (Figure 7A-B). Thus, the results revealed that DPN could inhibit the activity of ROCK in HSCs, but its capacity was less than that of the classic ROCK inhibitor Y-27632 (Figure 7E-F).

Effect of DPN on MLC activity in HSCs

We also investigated the phosphorylation of MLC in the five cell treatment groups. As a result, DPN played a more powerful role in inhibiting p-MLC (Thr^{18}/Ser^{19}) than inhibiting p-moesin (Thr^{558}). The role of DPN in

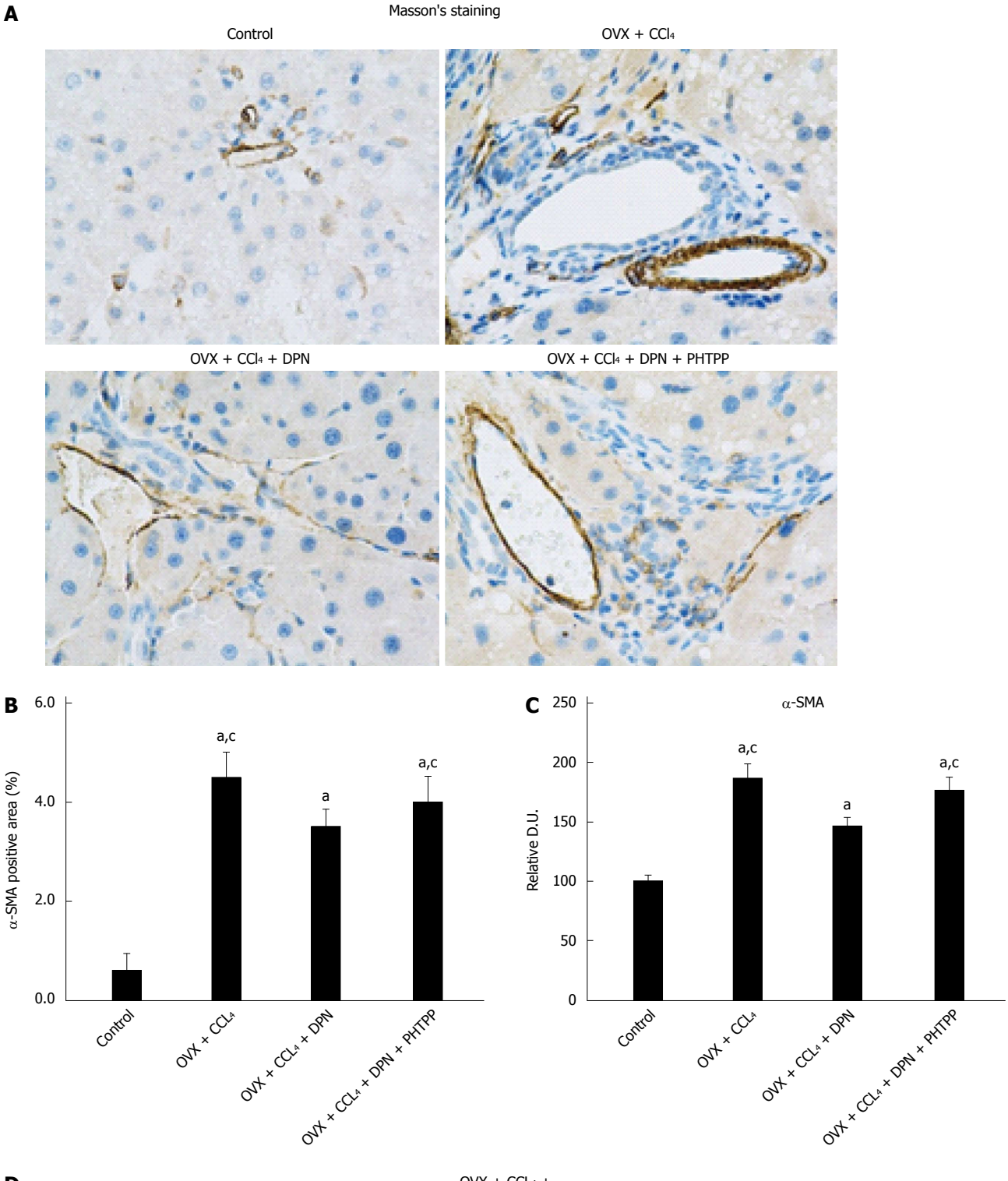


Figure 2 Diarylpropionitrile downregulates α-SMA expression in the livers of CCl_4-treated rats. A and B: Immunohistochemical staining for α-SMA (magnification × 400); C and D: Analysis of α-SMA protein expression by Western blot (each group $n = 5$). $^aP < 0.05$ *vs* control group; $^cP < 0.05$ *vs* DPN group. DPN: Diarylpropionitrile.

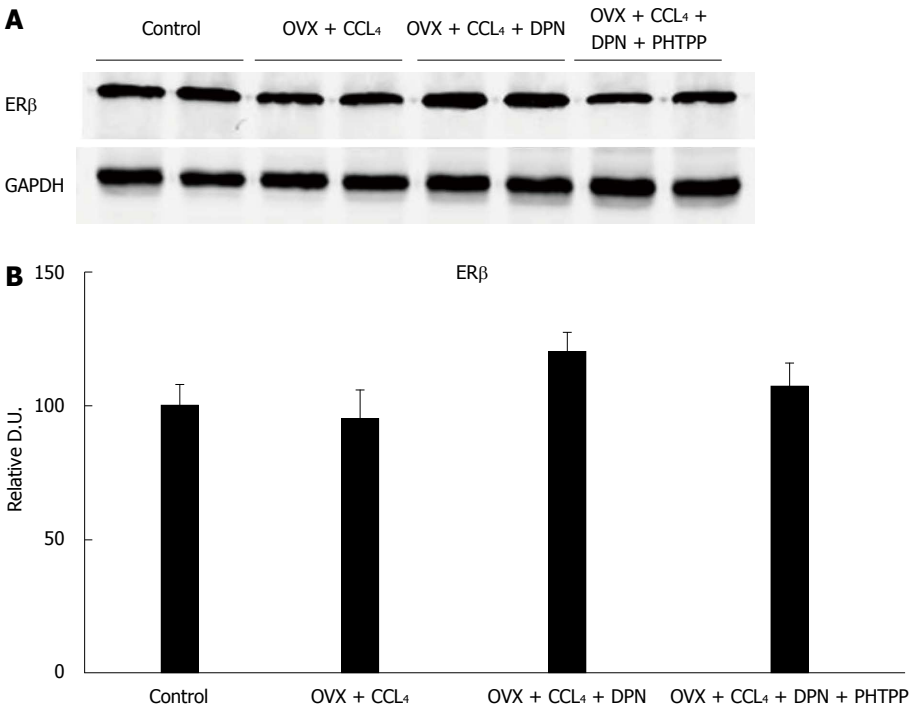


Figure 3 There were no statistically significant differences between the hepatic estrogen receptor β protein expression levels of all groups, as determined by Western blot analysis (A-B) (each group *n* = 5). [a]*P* < 0.05 *vs* control group; [c]*P* < 0.05 *vs* DPN group. DPN: Diarylpropionitrile; ERβ: Estrogen receptor β.

Table 2 *In vivo* hemodynamic data of the four treatment groups

Parameter	Control	OVX + CCl_4	OVX + CCl_4 + DPN	OVX + CCl_4 + DPN + PHTPP
PP (mmHg)	7.5 ± 1.1	14.9 ± 1.6[a,c]	11.0 ± 1.3[a]	14.6 ± 1.5[a,c]
CO (mL/min per 100 g)	19.3 ± 2.4	32.2 ± 5.0[a,c]	25.7 ± 4.0[a]	31.4 ± 5.4[a,c]
MAP (mmHg)	120.6 ± 14.2	82.1 ± 12.2[a]	95.7 ± 14.0[a]	89.5 ± 13.3[a]
TPR (mmHg/mL/min per 100 g)	6.0 ± 0.9	2.6 ± 0.3[a]	3.1 ± 0.7[a]	2.9 ± 0.6[a]
PVI (mL/min per 100 g)	2.1 ± 0.3	4.8 ± 0.8[a,c]	3.0 ± 0.4[a]	4.4 ± 0.6[a,c]
SVR (mmHg/mL/min per 100 g)	55.5 ± 8.5	15.0 ± 2.3[a,c]	31.7 ± 3.7[a]	16.8 ± 1.5[a,c]
PSS (%)	0.2 ± 0.1	50.5 ± 6.3[a,c]	27.9 ± 4.9[a]	48.1 ± 6.1[a,c]
IHVR (mmHg/mL/min per 100 g)	1.5 ± 0.2	3.2 ± 0.6[a,c]	2.2 ± 0.3[a]	3.0 ± 0.5[a,c]

[a]*P* < 0.05 *vs* control group; [b]*P* < 0.05 *vs* DPN group. OVX: Ovariectomized; SVR: Systemic vascular resistance; DPN: Diarylpropionitrile.

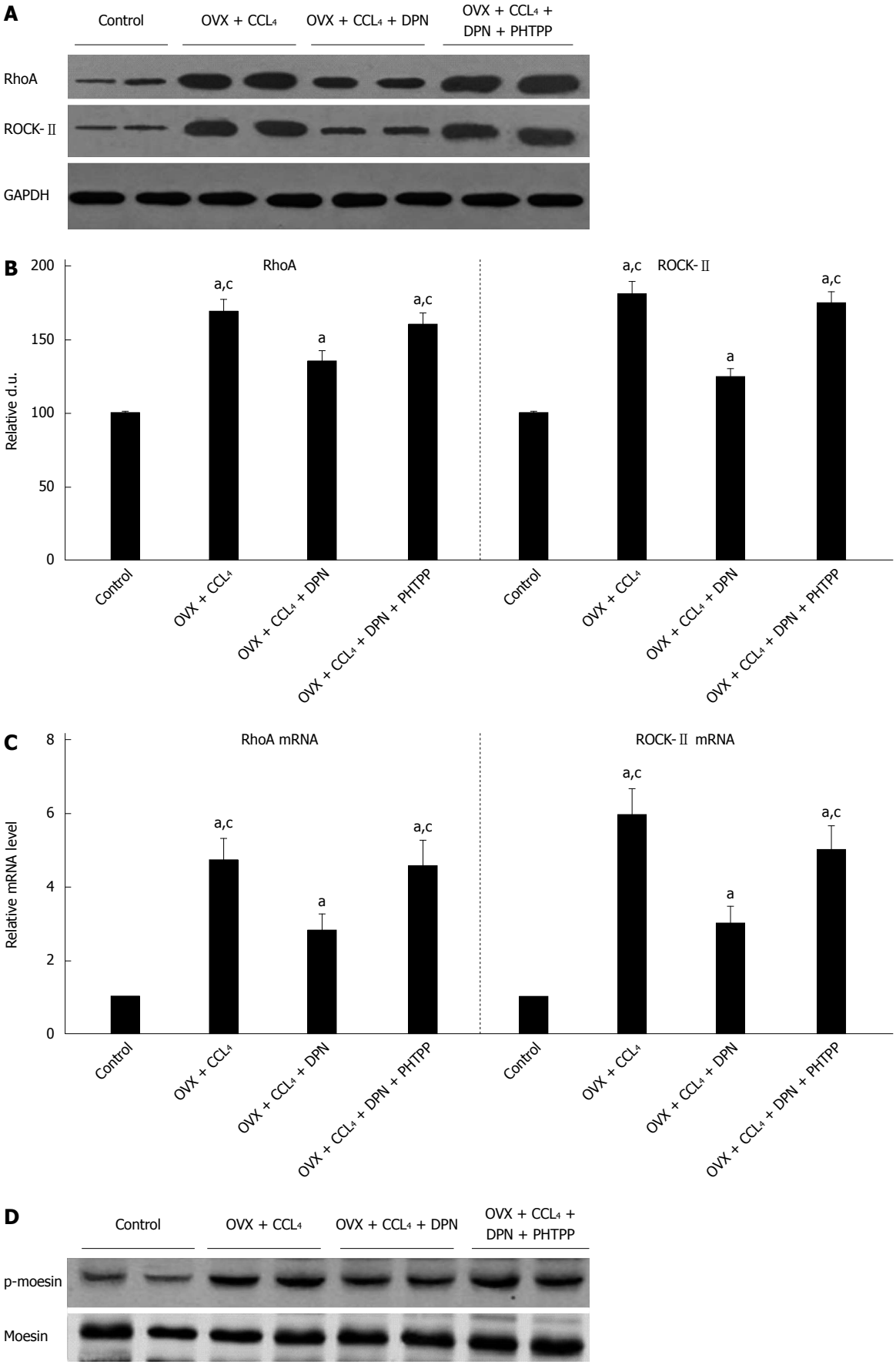
inhibiting p-MLC (Thr^{18}/Ser^{19}) was similar to that of Y-27632 (Figure 7E-F).

DISCUSSION

Our study shows that DPN, an ERβ selective agonist, not only postpones the development of liver cirrhosis, but also decreases the PP and IHVR in OVX CCl_4-induced cirrhotic rats. Furthermore, our data demonstrate that DPN inhibits the RhoA/ROCK pathway and activates the NO/PKG pathway, leading to the inactivation of p-MLC in the cirrhotic livers of OVX rats. Furthermore, the *in vitro* studies demonstrate that DPN suppresses the contraction of HSCs, which is associated with the inhibition of ROCK and phosphorylation of MLC.

In the previous studies, estrogen therapy was indicated to improve hepatic fibrosis[12-15]. Thus, we first examined the effect of DPN on CCl_4-induced liver cirrhosis in OVX rats by histological and immunochemical assessments. Furthermore, we examined α-SMA expression. HE staining and computerized collagen volume fraction analysis using trichrome staining indicated that DPN significantly inhibited CCl_4-induced liver cirrhosis in OVX rats (Figure 1). α-SMA expression markedly activated HSCs, which play a critical role in liver fibrogenesis[26]. Immunohistochemical staining and Western blot analysis against α-SMA confirmed that DPN downregulated α-SMA expression in CCl_4-induced cirrhotic livers. However, an ERβ antagonist, PHTPP, counteracted the effect of DPN (Figure 2). This indicates that DPN may improve liver cirrhosis in an HSC-dependent manner. The results of the liver and renal examinations affirmed the conclusion above (Table 1).

To investigate the effect of DPN on PP, IHVR and hyperdynamic circulation, we measured *in vivo* hemodynamic parameters using the microspheres


A
Control
OVX + CCl4
OVX + CCl4 + DPN
OVX + CCl4 + DPN + PHTPP
RhoA
ROCK-Ⅱ
GAPDH
B
RhoA
ROCK-Ⅱ
Relative d.u.
200
150
100
50
0
a,c
a
a,c
a,c
a
a,c
Control
OVX + CCl4
OVX + CCl4 + DPN
OVX + CCl4 + DPN + PHTPP
C
RhoA mRNA
ROCK-Ⅱ mRNA
Relative mRNA level
8
6
4
2
0
a,c
a
a,c
a,c
a
a,c
Control
OVX + CCl4
OVX + CCl4 + DPN
OVX + CCl4 + DPN + PHTPP
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Control
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p-moesin
Moesin

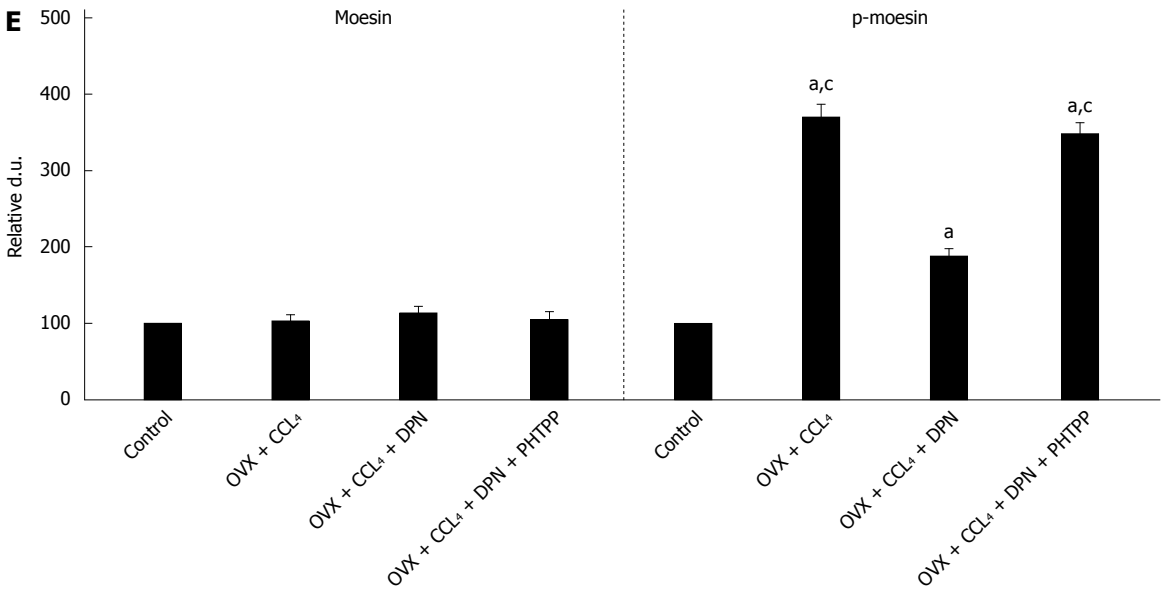


Figure 4 Diarylpropionitrile inhibits the protein (A, B) and mRNA (C, D) expression of RhoA and ROCKII, and even suppresses the site-specific phosphorylation of moesin (Thr^{558}) in CCl_4-treated rats (D, E). Shown are the relative densitometric quantifications of all experiments (mean ± SE), with values from the sham-operated controls set to 100 DU. $^aP < 0.05$ *vs* control group; $^cP < 0.05$ *vs* DPN group. DPN: Diarylpropionitrile.

technique. Interestingly, our data indicated that DPN markedly decreased the PP and IHVR in CCl_4-induced cirrhotic OVX rats, but PHTPP counteracted the effects of DPN. This suggested that DPN played an important role in decreasing the PP and IHVR *via* ERβ in the liver. Moreover, DPN improved the hyperdynamic circulation of cirrhotic rats without effecting the MAP or TPR (Table 2). In this regard, DPN is clearly different from other vasodilators, such as nitrates and ROCK antagonists, as these medicines have a risk of decreasing MAP and TRP[27,28]. This characteristic of DPN is worth intensively exploring.

Although the liver did not work as the estrogen's classic target organ, in our present experiment, Western blot analysis showed that there indeed existed ERβ expression in the livers of all of four rat groups. This result is consistent with a previous study[20], and is the basis of DPN having an effective role in the liver (Figure 3).

More and more evidence indicates that the high responsiveness of the intrahepatic vascular bed is closely related to increased expression and activation of ROCK in cirrhotic livers, which leads to increased IHVR[7]. Furthermore, ROCK antagonists, such as Y-27632 and fasudil, significantly decrease the IHVR[28]. As described in previous studies[29-31], estrogen could attenuate vascular contraction through inhibition of the RhoA/ROCK pathway. Thus, we further investigated the effect of DPN on the RhoA/ROCK pathway in the CCl_4-induced cirrhotic livers of rats. Our data indicate that treatment with DPN greatly reduced the mRNA and protein expression levels of RhoA and ROCKⅡ (Figure 4A-C). Moreover, DPN inhibited the increase in moesin phosphorylation typically seen in the cirrhotic livers, without altering the levels of total moesin (Figure 4D-E). This means that DPN not only inhibited the expression of RhoA and ROCK, but also blocked hepatic ROCK activity as seen by the suppression of moesin phosphorylation, which is a common measure of ROCK activity. Therefore, it can be said that besides improving liver fibrosis, DPN can decrease intrahepatic vasoconstriction, thus decreasing intrahepatic vascular tone.

RhoA/ROCK signaling and NO/PKG signaling regulated each other and maintained the balance between intrahepatic vasocontraction and vasodilatation[9-11]. Hence, we explored the effect of DPN treatment on NO/PKG signaling in the cirrhotic livers of OVX rats. It has been reported that although eNOS protein levels may appear unchanged, eNOS activity and NO production can decrease in sinusoidal endothelial cells (SECs)[16,32]. It was also been reported that estrogen stimulated eNOS expression in SECs and increased NO production in both normal and cirrhotic rats[32,33]. Our experiment revealed unchanged eNOS expression in cirrhotic OVX rats compared to the control group, although the levels of p-eNOS (Ser^{1177}) were noticeably lower (Figure 5). However, DPN might not only increase eNOS expression, but also increase p-eNOS (Ser^{1177}) levels (Figure 5). Hence, DPN could not only increase the expression of eNOS in SECs, but also upregulate the activation of eNOS, ultimately augmenting eNOS-derived NO generation. In addition, we investigated iNOS expression, and found that the markedly increased iNOS expression seen in the cirrhotic livers of OVX rats could be inhibited by DPN, which could in turn be blocked by PHTPP (Figure 5). It was reported that estrogen, which has potent antioxidant properties, could significantly attenuate cytokine-induced iNOS production in rat hepatocytes[34]. Thus, we speculate

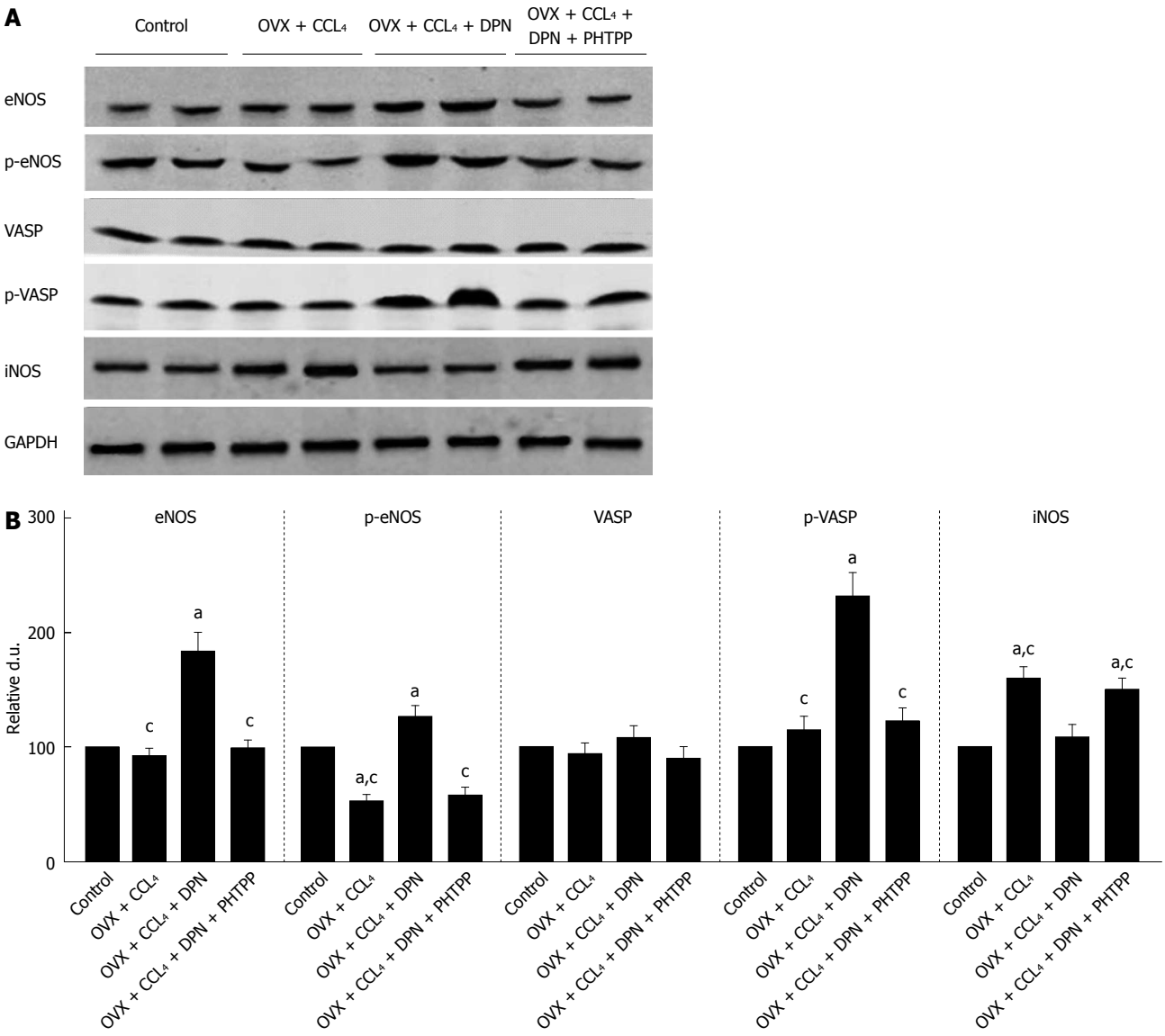


Figure 5 Diarylpropionitrile increases the hepatic expression of NO/PKG pathway proteins and increases their activity but inhibits hepatic iNOS expression in CCl_4-treated rats. A: Western blot analysis of eNOS, p-eNOS, VASP, p-VASP, and iNOS protein expression; B: Relative densitometric quantifications of all experiments (mean ± SE), with the values from the controls set to 100 DU (each group $n = 5$). $^aP < 0.05$ *vs* control group; $^cP < 0.05$ *vs* DPN group.

that DPN might negatively regulate protein nitrosylation and enhance NO bioavailability *via* inhibiting iNOS, which is closely associated with oxidative stress and cytokines[35,36]. To further understand the role of DPN in the activation of PKG, we detected phosphorylation levels of VSAP, an endogenous PKG substrate[8,22]. Our data indicate that DPN upregulates p-VASP (Ser239) and ultimately mediates NO-induced vasodilatation (Figure 5). There is evidence that defective eNOS signaling is mediated by ROCK activation in rats with secondary biliary cirrhosis[12], and that PKG-dependent RhoA deactivation would lead to a perpetuating loop in the effect of statins on RhoA/ROCK activity[37]. In our current study, we also found that inhibition of the RhoA/ROCK pathway might contribute to the activity of NO/PKG pathway in CCl_4-induced cirrhotic OVX rats, and vice versa. Taken together, we concluded that DPN could simultaneously play an important role in the inhibition of the RhoA/ROCK and the activation of the NO/PKG pathways in the intrahepatic vascular system of cirrhotic rats. In VSMCs, both inhibition of the RhoA/ROCK pathway and activation of the NO/PKG pathways commonly attribute to the activity of myosin light chain phosphatase (MLCP), causing inhibited myosin light chain phosphorylation and vasodilatation[38]. As both pathways converge at this step, the level of MLC phosphorylation was detected. The results indicated that treatment with DPN significantly decreased the level of p-MLC (Thr^{18}/Ser^{19}), however, PHTPP can offset this effect (Figure 5). In conclusion, it can be said that DPN attenuates vasoconstriction in the cirrhotic livers of OVX rats by inhibiting MLC activity *via* regulation of the RhoA/ROCK and NO/PKG pathways.

HSCs have crucial importance in the development of liver cirrhosis[26]. Activated HSCs transform into myofibroblast-like cells, and acquire contractility. Their

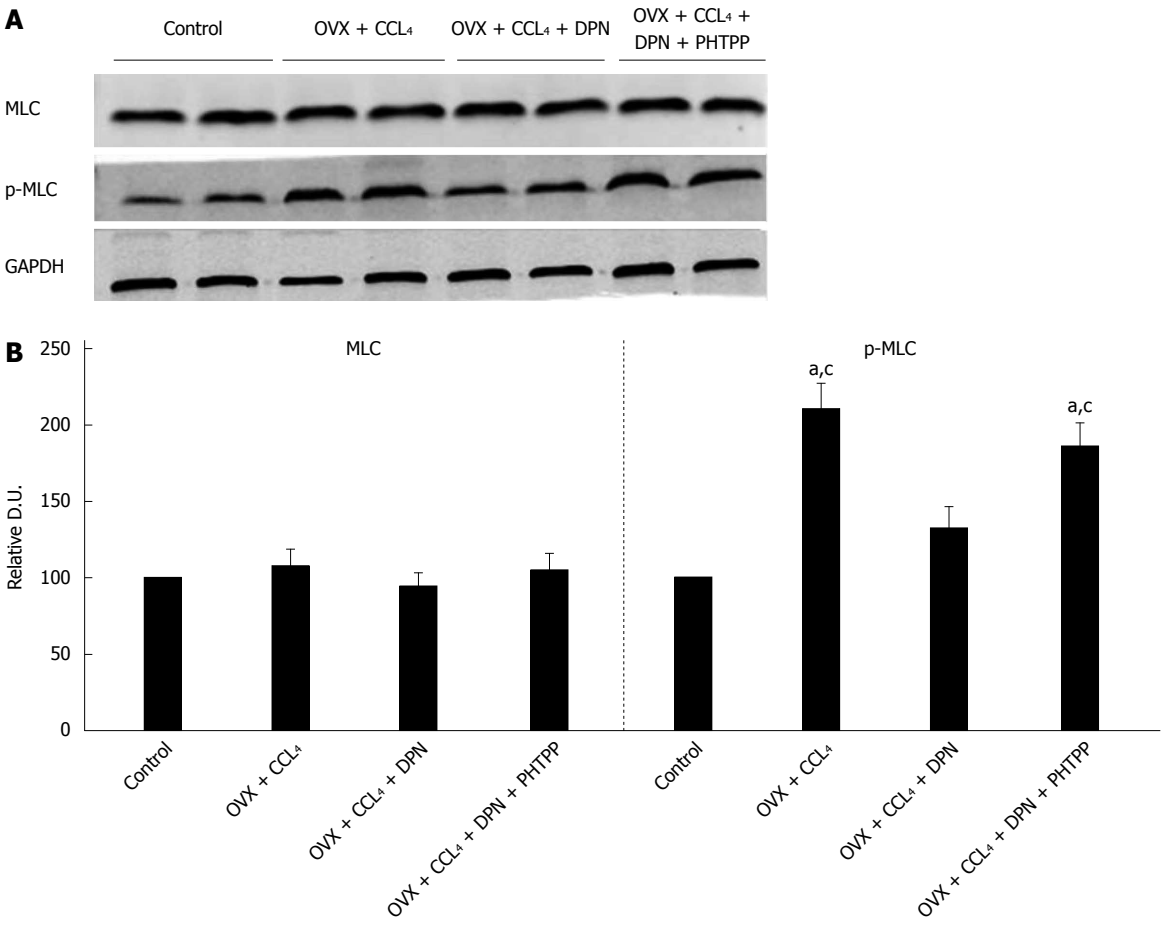
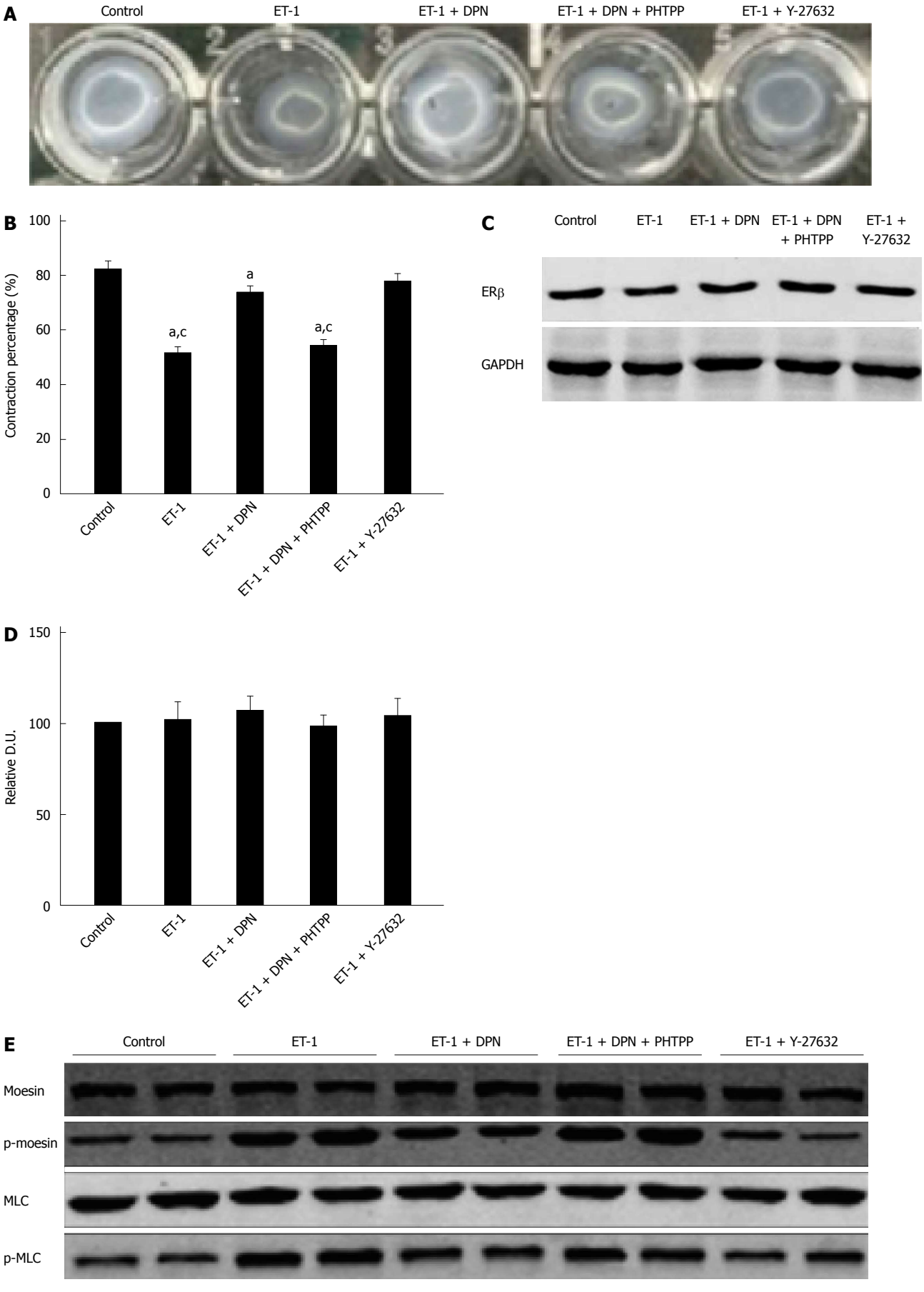


Figure 6 Diarylpropionitrile inhibits the phosphorylation of MLC in the livers of CCl_4-treated rats. A: Western blot analysis of total MLC and p-MLC; B: Relative densitometric quantifications of all experiments (mean ± SE), with the values of the controls set to 100 DU (each group $n = 5$) $^aP < 0.05$ *vs* control group; $^cP < 0.05$ *vs* DPN group. DPN: Diarylpropionitrile.

shrinkage is reported to be conditioned mainly through a Ca^{2+}-sensitization mechanism which is analogous to the contraction in VSMCs[39,40]. Therefore, HSCs were considered the key cells in IHVR regulation. Recently, selective inhibition of the RhoA/ROCK pathway in activated HSCs has been regarded as a potential novel therapeutic target to reduce PHT[41,42]. Our *in vivo* studies suggest that DPN treatment in OVX cirrhotic rats significantly reduced hepatic α-SMA expression, thus we hypothesized that DPN negatively regulates the activation of HSCs. Nonetheless, the effect of DPN on the contraction of HSCs required more extensive investigation. First, we verified that ERβ expression was truly present in HSCs (Figure 7C-D). With regard to the physiological role of DPN in the regulation of HSC contractility, a collagen gel contraction assay was performed. We observed that 10^{-7} mol/L DPN could significantly inhibit the 10^{-7} mol/L ET-1-induced contraction of the HSC containing gel lattices. This was also observed with 10^{-5} mol/L Y-27632, while 10^{-7} mol/L PHTPP counteracted the effect of DPN (Figure 7A-B). Hence, it can be said that 10^{-7} mol/L DPN has a similar efficacy to 10^{-5} mol/L Y-27632 in blocking the HSC contraction.

To further specify the role of DPN in the regulation of ROCK activity in HSCs, the phosphorylation of moesin was examined. Although 10^{-7} mol/L DPN was less effective than 10^{-5} mol/L Y-17632 in downregulating p-moesin (Thr^{558}), treatment with 10^{-7} mol/L DPN still significantly decreased p-moesin (Thr^{558}) levels compared to HSCs stimulated with 10^{-7} mol/L ET-1 (Figure 7D-E). We therefore concluded that DPN could be a novel ROCK inhibitor used to block the contraction of activated HSCs. To our knowledge, in HSCs the inactivation of MLCP is mainly regulated by the RhoA/ROCK pathway[43]. So we investigated the effect of DPN on the phosphorylation of MLC in HSCs. Interestingly, 10^{-7} mol/L DPN distinctly inhibited the ET-1-induced overexpression of p-MLC (Thr^{18}/Ser^{19}), and 10^{-7} mol/L DPN was equally effective in inhibiting p-MLC (Thr^{18}/Ser^{19}) as 10^{-5} mol/L Y-27632 (Figure 7D-E). Although DPN was less potent than Y-27632 in blocking the moesin phosphorylation (Thr^{558}), its final effect on p-MLC (Thr^{18}/Ser^{19}) and HSC contraction was no different than that of Y-27632. Further research is needed to fully explore the mechanisms involved. As a final note, it has been shown that HSCs contain functional ERβ but no ERα[20]; therefore, DPN could be used as a targeted ROCK inhibitor without causing severe systemic side effects.


A
Control
ET-1
ET-1 + DPN
ET-1 + DPN + PHTPP
ET-1 + Y-27632
B
Contraction percentage (%)
100
80
60
40
20
0
a,c
a
a,c
Control
ET-1
ET-1 + DPN
ET-1 + DPN + PHTPP
ET-1 + Y-27632
C
Control
ET-1
ET-1 + DPN
ET-1 + DPN + PHTPP
ET-1 + Y-27632
ERβ
GAPDH
D
Relative D.U.
150
100
50
0
Control
ET-1
ET-1 + DPN
ET-1 + DPN + PHTPP
ET-1 + Y-27632
E
Control
ET-1
ET-1 + DPN
ET-1 + DPN + PHTPP
ET-1 + Y-27632
Moesin
p-moesin
MLC
p-MLC

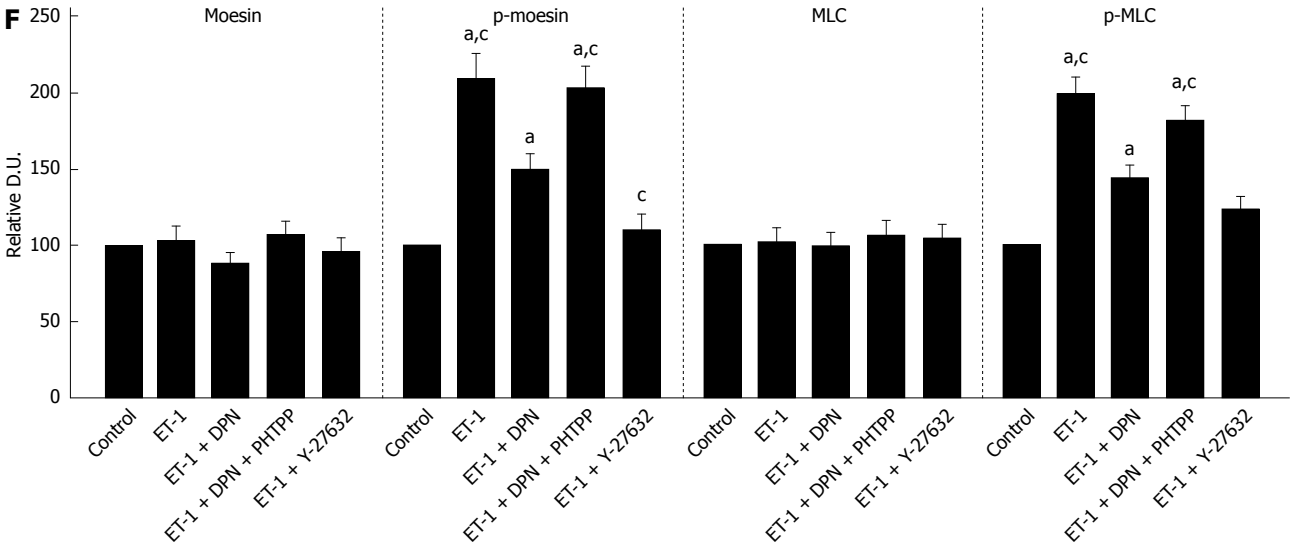


Figure 7 Diarylpropionitrile inhibits collagen lattice contraction in hepatic stellate cells and decreases ET-1 induced moesin and MLC phosphorylation. A: Appearance of collagen lattices 4 h after drug treatment; B: The percentage of remaining lattice area 4 h after drug treatment (each group n = 12); C, D: Western blot analysis of ERβ protein expression in hepatic stellate cells (HSCs) (each group n = 5); E: Western blot analysis of the total and phosphorylated moesin and MLC in HSCs; F: Relative densitometric quantifications of moesin and MLC experiments (mean ± SE), with the values of the controls set to 100 DU (each group n = 5). $^{a}P < 0.05$ *vs* control group; $^{c}P < 0.05$ *vs* DPN group. OVX: ovariectomized; SVR: Systemic vascular resistance; DPN: Diarylpropionitrile.

In summary, treatment with DPN was effective in lowering PHT in CCl_4-induced cirrhotic OVX rats, which was attributed to its anti-hepatic fibrosis effect and its ability to decrease IHVR *via* inhibition of the RhoA/ROCK and activation of the NO/PKG signaling pathways. DPN also significantly reduced HSC contractility by inhibiting ROCK activation and downstream MLC phosphorylation. This study suggests that DPN could be a potential candidate for estrogen replacement therapy, benefiting menopausal women with liver cirrhosis and PHT.

COMMENTS

Background

Increased intrahepatic vascular resistance (IHVR) is a major cause for portal hypertension (PHT), and activated hepatic stellate cells (HSCs), contraction of intrahepatic vascular smooth muscle cells, and reduced vasodilator nitric oxide levels all play a critical role in contributing to increased IHVR. NO is of great significance in increasing IHVR levels. Animal experiments and clinical trials provide consistent evidence for the protective effect of endogenous and exogenous estrogens on liver fibrosis. However, exogenous estrogens give rise to a number of potential risks, which restrain them from clinical uses.

Research frontiers

Evidence indicates the intrahepatic upregulation of RhoA and Rho-kinase signaling and inhibition of NO/PKG signaling from increasing IHVR. For these reasons, the two pathways serve as the crucial therapeutic target to ameliorate PHT. High estrogen receptor (ER) β expression levels and low ERα expression levels were observed in livers, moreover, HSCs have functional ERβ, rather than ERα. Therefore, this paper studied the effect of DPN - an ERβ selective agonist - on the two pathways, and also on hepatic hemodynamics systemically.

Innovations and breakthroughs

DPN treatment is effective in lowering PHT in CCl_4-induced cirrhosis of the OVX rats, contributes to its anti-hepatic fibrosis effect, and is capable of decreasing IHVR by the inhibition of RhoA/ROCK and activation of the NO/PKG signaling pathways. DPN also significantly reduced HSC shrinkage by restraining ROCK activation and down-streaming MLC phosphorylation *via* ERβ.

Applications

The ERβ selective agonist may be a potential therapeutic approach to managing PHT and liver fibrosis, particularly for those menopausal women and patients with low estrogen levels.

Terminology

ER subtypes play a distinct role in exerting different biological effects with tissue-specific responses. ERβ selective agonists may produce biological effects without causing any classic side effects of estrogens.

Peer-review

This is a well-done experimental study concerning the efficacy of ER agonist against cirrhosis-related portal hypertension. Clinically practical benefits will not come into effect within a short time. However, the study suggests that the ERβ selective agonist be a potential therapeutic method to manage PHT and liver fibrosis.

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ORIGINAL ARTICLE

Basic Study

miR-30b inhibits autophagy to alleviate hepatic ischemia-reperfusion injury *via* decreasing the Atg12-Atg5 conjugate

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Author contributions: Li SP, Wang Z, and Yu Y performed the majority of experiments and analyzed the data; He JD, Wang Z, and Yu Y performed the molecular investigations; He JD, Wang Z, and Yu Y participated equally in treatment of animals; Zhang HM, Zhang JJ, and Shen ZY designed and coordinated the research; Li SP, Fu SY, and Zhang JJ wrote the paper; Li SP, He JD, Wang Z, Yu Y, and Fu SY contributed equally to this work.

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Institutional animal care and use committee statement: All animals were housed and handled according to Tianjin Medical University Institutional Animal Care and Use Committee guidelines, and all animal work was approved by the appropriate committee.

Conflict-of-interest statement: There is no conflict of interest in this study.

Data sharing statement: No additional data are available.

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Abstract

AIM: To explore the role and potential mechanism of miR-30b regulation of autophagy in hepatic ischemia-reperfusion injury (IRI).

METHODS: An animal model of hepatic IRI was generated in C57BL/6 mice. For *in vitro* studies, AML12 cells were immersed in mineral oil for 1 h and then cultured in complete Dulbecco's Modified Eagle's Medium

(DMEM)/F12 to simulate IRI. Mice and cells were transfected with miR-30b agomir/mimics or antagomir/inhibitor to examine the effect of miR-30b on autophagy to promote hepatic IRI. The expression of miR-30b was measured by real-time polymerase chain reaction. Apoptotic cells were detected by terminal uridine nick-end labeling (TUNEL) staining, and cell viability was detected by methylthiazole tetrazolium assay. The expression of light chain 3, autophagy-related gene (Atg)12, Atg5, P62, and caspase-3 were detected by western blotting analysis.

RESULTS: miR-30b levels were significantly down-regulated after hepatic IRI, and the numbers of autophagosomes were increased in response to IRI both *in vivo* and *in vitro*. These findings demonstrate that low levels of miR-30b could promote hepatic IRI. Furthermore, we found that miR-30b interacted with Atg12-Atg5 conjugate by binding to Atg12. Overexpression of miR-30b diminished Atg12 and Atg12-Atg5 conjugate levels, which promoted autophagy in response to IR. In contrast, downregulation of miR-30b was associated with increased Atg12-Atg5 conjugate levels and increased autophagy.

CONCLUSION: miR-30b inhibited autophagy to alleviate hepatic ischemia-reperfusion injury *via* decreasing the Atg12-Atg5 conjugate.

Key words: miR-30b; Autophagy; Atg12-Atg5 conjugate; Hepatic ischemia-reperfusion injury

Core tip: miR-30b levels were significantly down-regulated after hepatic ischemia-reperfusion injury (IRI) in mice. The number of autophagosomes was increased in response to IRI both *in vivo* and *in vitro*. Decreased levels of miR-30b could promote hepatic IRI, as revealed by reductions in cells viability *in vitro*. Overexpression of miR-30b diminished autophagy-related gene (Atg)12 and Atg12-Atg5 conjugate levels which promoted autophagy in response to hepatic IRI. Therefore, miR-30b inhibits autophagy to alleviate hepatic IRI *via* decreasing the Atg12-Atg5 conjugate.

Li SP, He JD, Wang Z, Yu Y, Fu SY, Zhang HM, Zhang JJ, Shen ZY. miR-30b inhibits autophagy to alleviate hepatic ischemia-reperfusion injury *via* decreasing the Atg12-Atg5 conjugate. *World J Gastroenterol* 2016; 22(18): 4501-4514 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i18/4501.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i18.4501

INTRODUCTION

Hepatic ischemia-reperfusion injury (IRI) is an important factor for the prognosis of surgical outcomes and patient survival as well as the protection of hepatic cells[1]. A key consideration regarding liver function was revealed from a recent study demonstrating that autophagy represents a principal component of hepatology[2]. Autophagy consists of a tightly regulated intracellular catabolic pathway involving lysosomal degradation of cytoplasmic organelles and proteins[3]. miRNAs are closely linked to virtually all fundamental biological pathways[4], and they play critical roles in a broad range of biological processes including proliferation, differentiation, apoptosis, and stress responses[5].

Recent findings have indicated some novel roles for miRNAs in the regulation of autophagy[6,7]. In this report, we focused on miRNAs with direct autophagic implications, as exerted either through putative core components of autophagy machinery or through less well-characterized mechanisms. The 3′-untranslated region (UTR) of the autophagy associated gene 12 (Atg12) contains the predicated target sites for miRNA-30b (miR-30b), which were identified by luciferase reporter gene assays. The possibility exists that miR-30b might contribute to alleviating IRI *via* modulating autophagy through targeting Atg12. In this study, we attempted to determine whether miR-30b modulates autophagy and thus alleviate hepatic IRI. Specifically, we upregulated or downregulated expression of miR-30b to examine the effects of miR-30b on Atg12 and Atg12-Atg5 conjugate levels that regulate autophagy in hepatic IRI. Our data indicate that miR-30b might serve as a novel therapeutic target regulating autophagy in hepatic IRI.

MATERIALS AND METHODS

Animals and cell line

Male C57BL/6 mice (7-8-wk-old, 23 ± 3 g) were purchased from the experimental animal center of the PLA Military Medical Science Academy. All animals received humane care according to established standards and were maintained in an air-conditioned animal room at 25 ℃ with free access to water and food. All protocols conformed to the National Institute of Health (NIH) guidelines and all animals received care in compliance with the principles of laboratory animal care. The AML12 cell line (mouse hepatic cell) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, United States). The study was performed according to Tianjin Medical University Institutional Review Board guidelines, and the protocol was approved by the Institutional Review Board.

Reagents and antibodies

Dulbecco's Modified Eagle's Medium (DMEM)/F12 medium and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, United States); miR-30b-5p mimics/agomir, miR-30b-5p inhibitor/antagomir,

miR-NC, Atg12 siRNA and RiboFECTTM CP Reagent were purchased from RiboBio Co., Ltd. (Guangzhou, China); rapamycin and 3-MA were purchased from Selleck Inc (Houston, TX, United States). The In Situ Cell Death Detection Kit, TMR red and SYBR Green quantitative real time polymerase chain reaction (qRT-PCR) Master Mix were purchased from Roche Diagnostics GmbH (Mannheim, Germany); Trizol and Lipofectamine 2000 were obtained from Invitrogen (Carlsbad, CA, United States). Antibody Atg12, light chain 3 (LC3), P62, caspase-3, cleave caspase-3, poly ADP-ribose polymerase 1 (PARP1), β-actin, and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Cell Signaling Technology Inc (Beverly, MA, United States).

Animal model and treatment

The segmental (70%) hepatic ischemia model was performed as previously described[1]. There were six mice in the sham group, and the 24 mice in the IR group were divided by reperfusion times, consisting of 2, 6, 12, and 24 h. Mice in the miR-30b-5p agomir group (n = 6), miR-30b-5p antagomir group (n = 6), and miR-NC group (n = 6) received miR-30b-5p agomir (10 nmol/L), antagomir (10 nmol/L), or miR-NC (10 nmol/L), respectively, by tail intravenous injection 24 h prior to ischemia.

Serology detection

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels of the mice in all groups were determined with use of a commercial assay kit (Nanjing Jiancheng Biological Technology, Nanjing, China). Enzyme activities were expressed as international units per liter (U/L).

Histology and transmission electron microscopy

Samples of liver were fixed in 4% mediosilicic isotonic formaldehyde for 24 h, dehydrated, and embedded in paraffin. Five micrometer-thick sections were cut from each paraffin embedded tissue and stained with hematoxylin and eosin (HE) to evaluate the degree of liver damage. In addition, liver and cell samples were placed in 1% glutaraldehyde and post-fixed with 2% osmium tetroxide. The cell pellets or sections were embedded in epon resin. The data were quantified by counting the number of autophagosomes per cross-sectioned cell.

Cell culture and treatment

AML12 cells were plated at a density of 2 × 10^5 cells/mL in 6-well plates and divided into seven groups[8]: (1) Control group, cells were cultured in DMEM/F12 without treatment; (2) IR group, cells were immersed in mineral oil (1 mL/well) for 1 h to simulate ischemia then cultured in DMEM/F12 for 12 h to simulate reperfusion; (3) miR-30b-5p mimics group, cells were transfected with 50 nmol/L miR-30b-5p mimics or miR-NC using RiboFECTTM CP Reagent for 24 h according to the manufacturer's protocol, followed by reperfusion as described above; (4) miR-30b-5p inhibitor group, cells were transfected with 50 nmol/L miR-30b-5p inhibitor or miR-NC, followed by reperfusion; (5) Atg12 siRNA group, cells were treated with Atg12 siRNA or siRNA-NC for 24 h, followed by reperfusion; (6) Rapamycin group, cells were treated with 40 nmol/L rapamycin for 2 h, followed by reperfusion; and (7) 3-MA group, cells were treated with 60 μM 3-MA for 2 h, followed by reperfusion.

Methylthiazole tetrazolium bioassay

Cells were seeded onto 96-well plates (5 × 10^4 cells/well) and after culture for 24 h at 37 ℃, subjected to reperfusion as described above. Fresh medium was then added to each well together with 20 μL methylthiazole tetrazolium solution (5 mg/mL), and the plate was incubated at 37 ℃ for 4 h. The medium was then removed, and 200 μL dimethylsulfoxide was added per well. The optical density of each well was determined with a test wavelength of 490 nm.

Confocal fluorescent microscopic detecting autophagy

AML12 cells were cultured in 6-well plates to 60%-70% confluence. The cells were transfected with tandem GFP-RFP-LC3 adenovirus (Hanbio, Shanghai, China) according to the GFP-RFP-LC3 instruction manual to further confirm autophagy induction.

Immunocytochemistry

The streptavidin-peroxidase staining technique was used to detect protein following antigen retrieval by microwave treatment. After blocking endogenous peroxidase activity by incubating in 3% H_2O_2 for 15 min, specimens were incubated with antibodies [proliferating cell nuclear antigen (PCNA) and caspase-3] at 4 ℃ overnight. Specimens were incubated at room temperature for 1 h with the secondary antibody, then diaminobenzidine solution was used. Counterstaining was performed with hematoxylin.

qRT-PCR

Total RNA was isolated by Trizol and 1 μg of RNA for reverse transcription was prepared as described above. RT-PCR was performed in a total volume of 25 μL reaction mixture. U6 or GAPDH was used as an internal control and the expression levels was calculated using MxPro software (Version 4.0, Stratagene, La Jolla, CA, United States).

Luciferase reporter gene assays

The miRWalk database was used to predict the binding site on the 3'-UTR of miR-30b, and this database combines several bioinformatic platforms including TargetScan 4.2, miRBase, and miRanda. Luciferase reporter gene assay was performed using the Dual-Luciferase Reporter Assay System (Promega, Madison,

WI, United States) according to the manufacturer's instructions[9-11]. Cells were transferred into 24-well plates at 3 × 10^4 cells/well. After 24 h, the cells were transiently co-transfected with pRL-TK plasmid (Promega), and various constructs containing different lengths of the Atg12 5′-flanking region or pGL3-Basic. The luciferase activities were measured according to the manufacturer's instructions.

Western blot analysis

Protein samples were harvested from mice livers and AML12 cells. The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to the nitrocellulose membranes. Membranes were probed with the antibodies to Atg12, LC3, P62, caspase-3, cleave caspase-3, RARP1 and β-actin. Bound antibodies were then visualized using an enhanced chemiluminescence (ECL) detection kit with an appropriate HRP-conjugated secondary antibody.

Apoptosis analysis using terminal uridine nick-end labeling

Terminal uridine nick-end labeling (TUNEL) reactions were performed using an In Situ Cell Death Detection Kit, TMR red. For quantification, the mean number of TUNEL-positive cells, as determined using 200 ×, in five different fields was determined.

Statistical analysis

All data are presented as mean ± SD. Differences among groups were analyzed using a one-way analysis of variance followed by the Student-Newman-Keuls post-hoc test. The SPSS software 19.0 (Armonk, NY, United States) was used for these analyses, where $P < 0.05$ was considered to be statistically significant.

RESULTS

Hepatic IRI alters miR-30b, Atg12, and Atg5 mRNA expression in mice livers

With the IR mouse model, miR-30b-5p expression levels gradually decreased ($P < 0.05$) after reperfusion, however, levels of Atg12 and Atg5 mRNA increased thereafter ($P < 0.05$), as compared with the Sham group (Figure 1A).

Alterations of autophagy in mice livers induced by IRI

As shown in Figure 1B, the expression of Atg12, Atg12-Atg5 conjugate, and LC3II was upregulated as a function of time following reperfusion ($P < 0.05$). Meanwhile, cleave caspase-3 expression increased as a function of time after reperfusion.

Alterations of liver pathological changes and serum AST and ALT levels

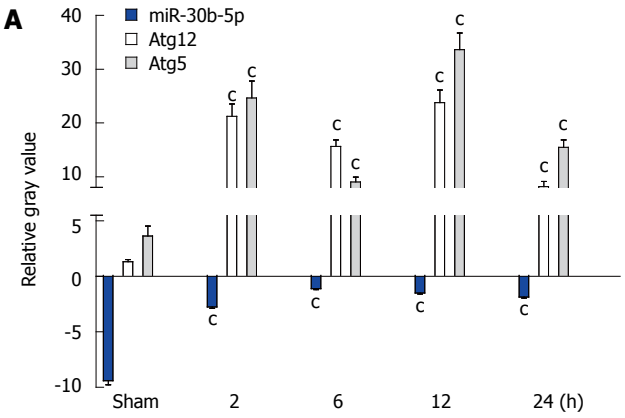
Pathological analyses as presented in Figure 1C, revealed considerable hepatocyte edema, congestion and apoptosis at 6-24 h post-reperfusion as compared with the Sham group. The time-dependent changes in serum AST and ALT levels of mice are illustrated in Figure 1D. Serum AST and ALT levels gradually increased ($P < 0.05$), reaching a peak at 12 h ($P < 0.001$) following reperfusion. Next, we evaluated autophagic vacuoles using Transmission Electron Microscopy (TEM). Autophagosomes, which contained partially degraded cytoplasmic material, were clearly observed with TEM (Figure 1E). The basal number of autophagosomes within the IR group was increased relative to the Sham group ($P < 0.001$).

miR-30b can alleviate mouse hepatic IRI

To clarify whether miR-30b can alleviate hepatic IRI, we either upregulated or downregulated the expression of miR-30b in mice after tail intravenous injection of miR-30b-5p agomir or antagomir, respectively, at 12 h following reperfusion. As shown in Figure 2A, we found that miR-30b-5p agomir significantly decreased the histopathologic changes of liver induced by IR treatment, but miR-30b-5p antagomir aggravated these changes. Compared with the miR-NC group, serum AST and ALT levels in the miR-30b-5p agomir group mice were decreased while those of the miR-30b-5p antagomir group mice were increased as a function of time following reperfusion ($P < 0.05$, Figure 2B). As illustrated in Figure 2C, the number of TUNEL-positive cells was significantly decreased compared with that of the miR-NC group ($P < 0.05$); however, miR-30b-5p antagomir increased the number of TUNEL-positive cells compared with that of the miR-NC group ($P < 0.01$). When compared with the miR-NC group at 12 h reperfusion, the miR-30b-5p agomir resulted in a significant increase in PCNA expression but a decrease in caspase-3, cleave caspase-3, and PARP1 expression. In contrast, the miR-30b-5p antagomir decreased in PCNA expression but increased in caspase-3, cleave caspase-3, and PARP1 expression (Figure 2D and E). These findings demonstrate that miR-30b can alleviate hepatic IRI.

miR-30b inhibits autophagy by sequestering Atg12

Based upon information contained within the bioinformatics database, we hypothesized that the miR-30b binding site was at the 3′-UTR of Atg12, and a luciferase reporter assay was performed to determine the effects of miR-30b on the 3′-UTR of Atg12 (Figure 3A). When examined at 12 h post-reperfusion, overexpression of miR-30b with miR-30b-5p mimics in AML12 cells significantly reduced Atg12 mRNA and protein levels compared with those cells transfected with the miR-NC ($P < 0.05$, Figure 3B). Moreover, levels of Atg12-Atg5 conjugate and LC3II decreased after transfection of miR-30b-5p mimics when compared with that of miR-NC ($P < 0.05$, Figure 3C). In contrast, the results in AML12 cells treated with miR-30b-5p inhibitor were opposite. Our findings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miR-30b-5p
Atg12
Atg5
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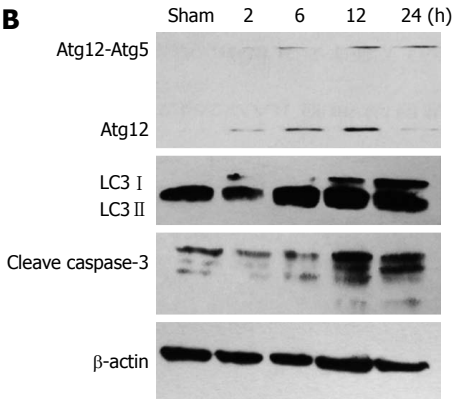
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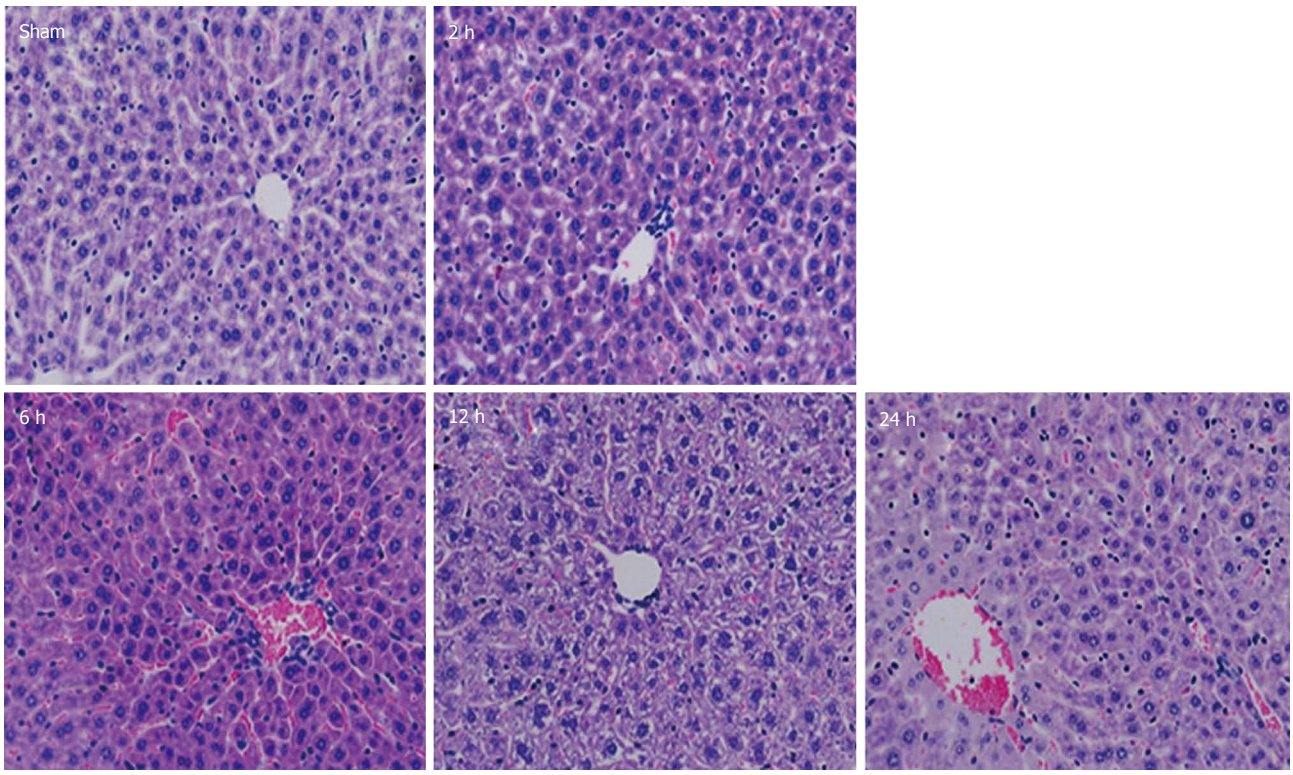
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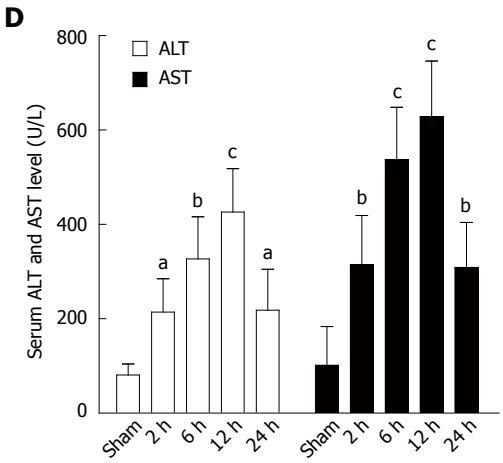
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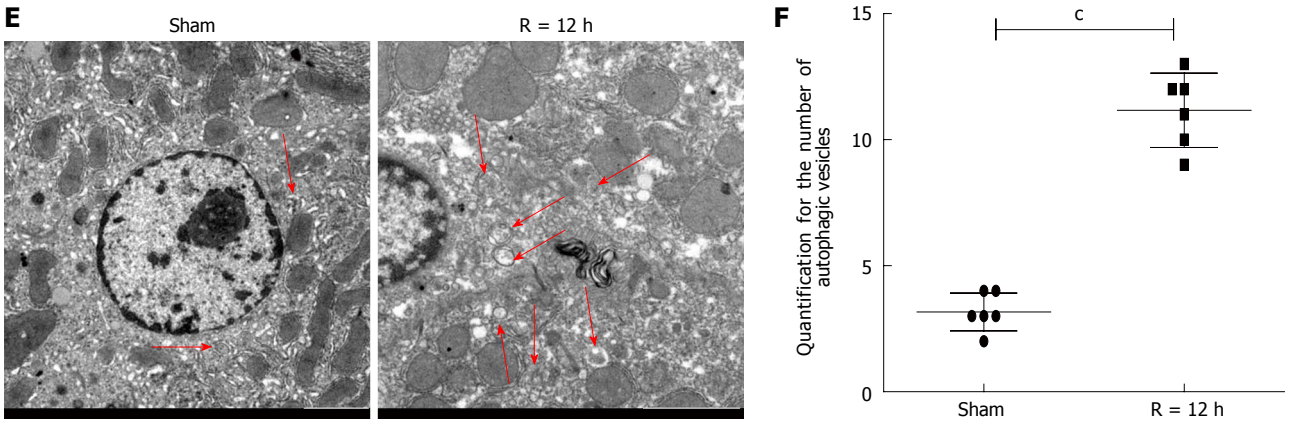
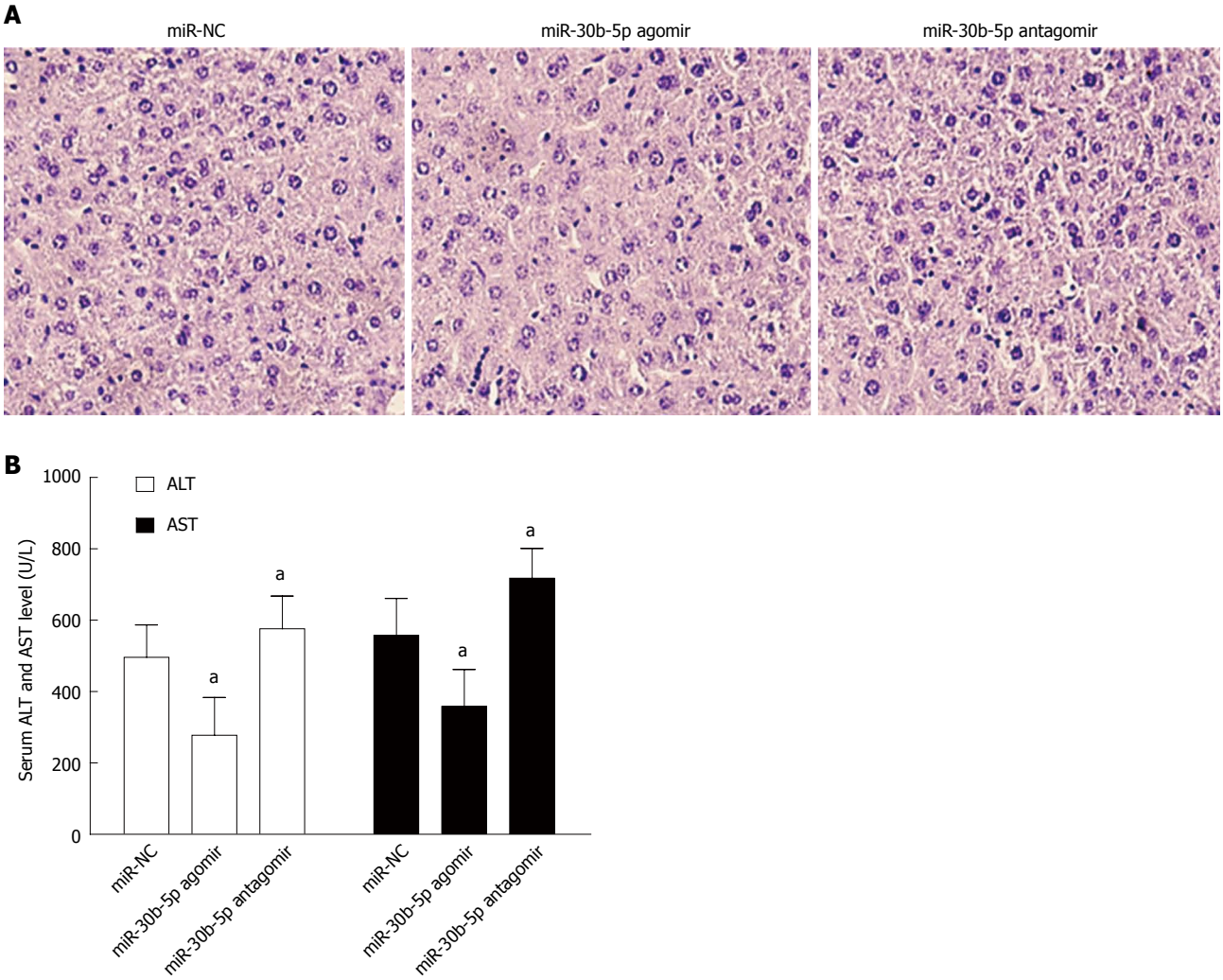


Figure 1 Alterations of miR-30b and autophagy in mouse livers in response to ischemia-reperfusion injury. A: The expression of miR-30b in mouse livers subjected to IR as determined by quantitative real time polymerase chain reaction analysis; B: Western blotting for autophagy-related gene (Atg) 12, light chain 3 (LC3), and cleave caspase-3 in mouse liver; C: IR treatment increases the histopathologic changes in liver (magnification × 200); D: IR treatment increased serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in mice compared with sham; E: Transmission electron microscopy (TEM) images of mouse hepatocytes after ischemia followed by reperfusion at 12 h. Scale bars = 2.0 μm. The data were quantified by counting the number of autophagosomes per cross-sectioned cell. $^{a}P < 0.05$,$^{b}P < 0.01$,$^{c}P < 0.001$ *vs* Sham group. Every experiment was repeated three times.



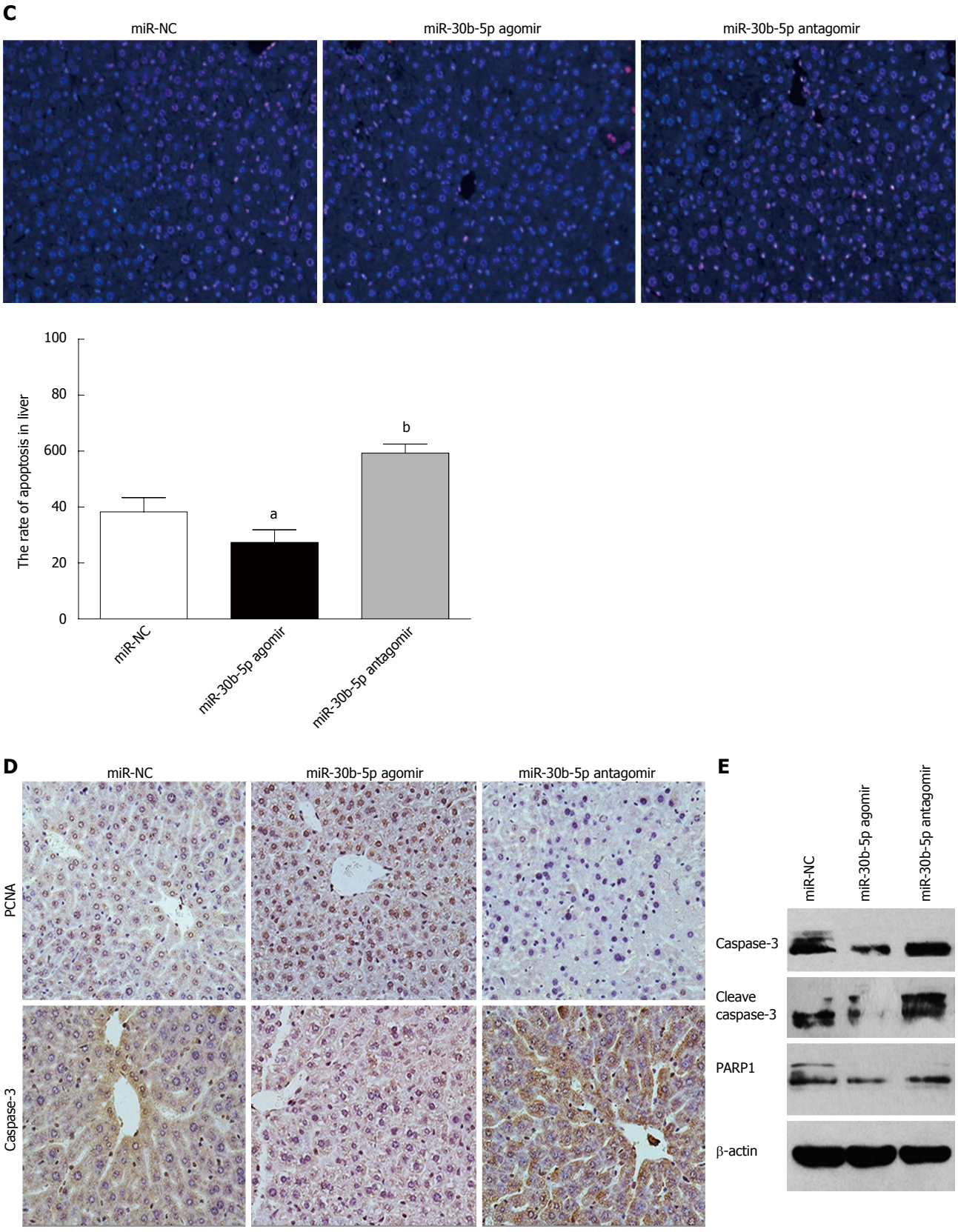
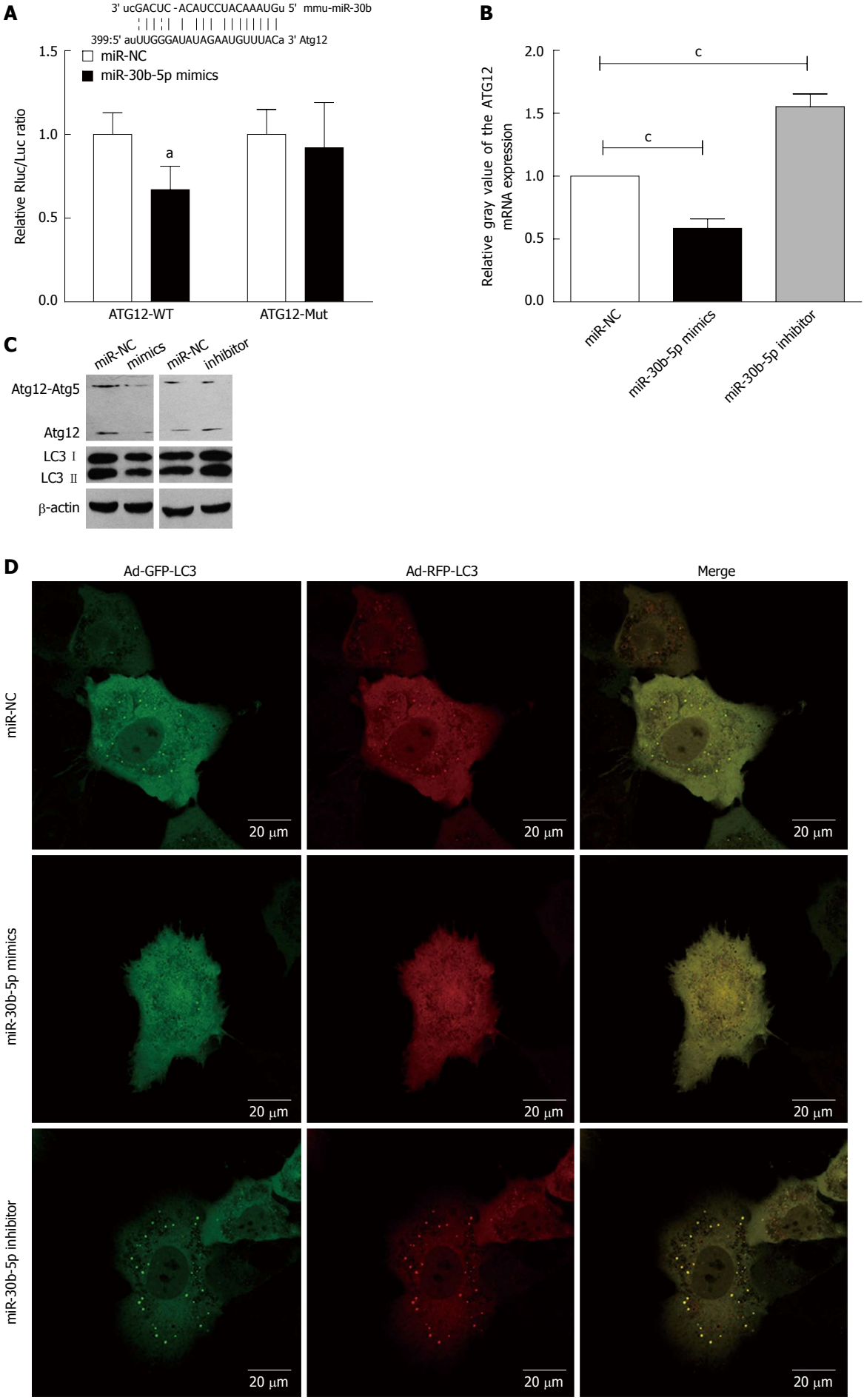


Figure 2 miR-30b can alleviate mouse hepatic ischemia-reperfusion injury. A: Hematoxylin and eosin (HE) stain was used to observe histopathologic changes in mouse liver (magnification × 200) after tail intravenous injection of miR-30b-5p agomir or antagomir at 12 h following reperfusion; and B: serum AST and ALT levels; C: Cell apoptosis as measured by terminal uridine nick end labeling (TUNEL). Representative sections as determined at 12 h post-reperfusion (magnification × 200); D: Immunohistochemistry revealed expression of proliferating cell nuclear antigen (PCNA) and caspase-3 (magnification × 200); E: Western blot was used to detect expression of caspase-3, cleave caspase-3, and poly ADP-ribose polymerase 1 (PARP1) in livers, $^{a}P < 0.05$,$^{b}P < 0.01$ *vs* miR-NC group. Every experiment was repeated three times.


A
3' ucGACUC - ACAUCCUACAAAUGu 5' mmu-miR-30b
399:5' auUUGGGAUAUAGAAUGUUUACa 3' Atg12
miR-NC
miR-30b-5p mimics
Relative Rluc/Luc ratio
1.5
1.0
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a
ATG12-WT
ATG12-Mut
B
Relative gray value of the ATG12 mRNA expression
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1.5
1.0
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c
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miR-NC
miR-30b-5p mimics
miR-30b-5p inhibitor
C
miR-NC
mimics
miR-NC
inhibitor
Atg12-Atg5
Atg12
LC3 I
LC3 II
β-actin
D
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Ad-RFP-LC3
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miR-30b-5p inhibitor
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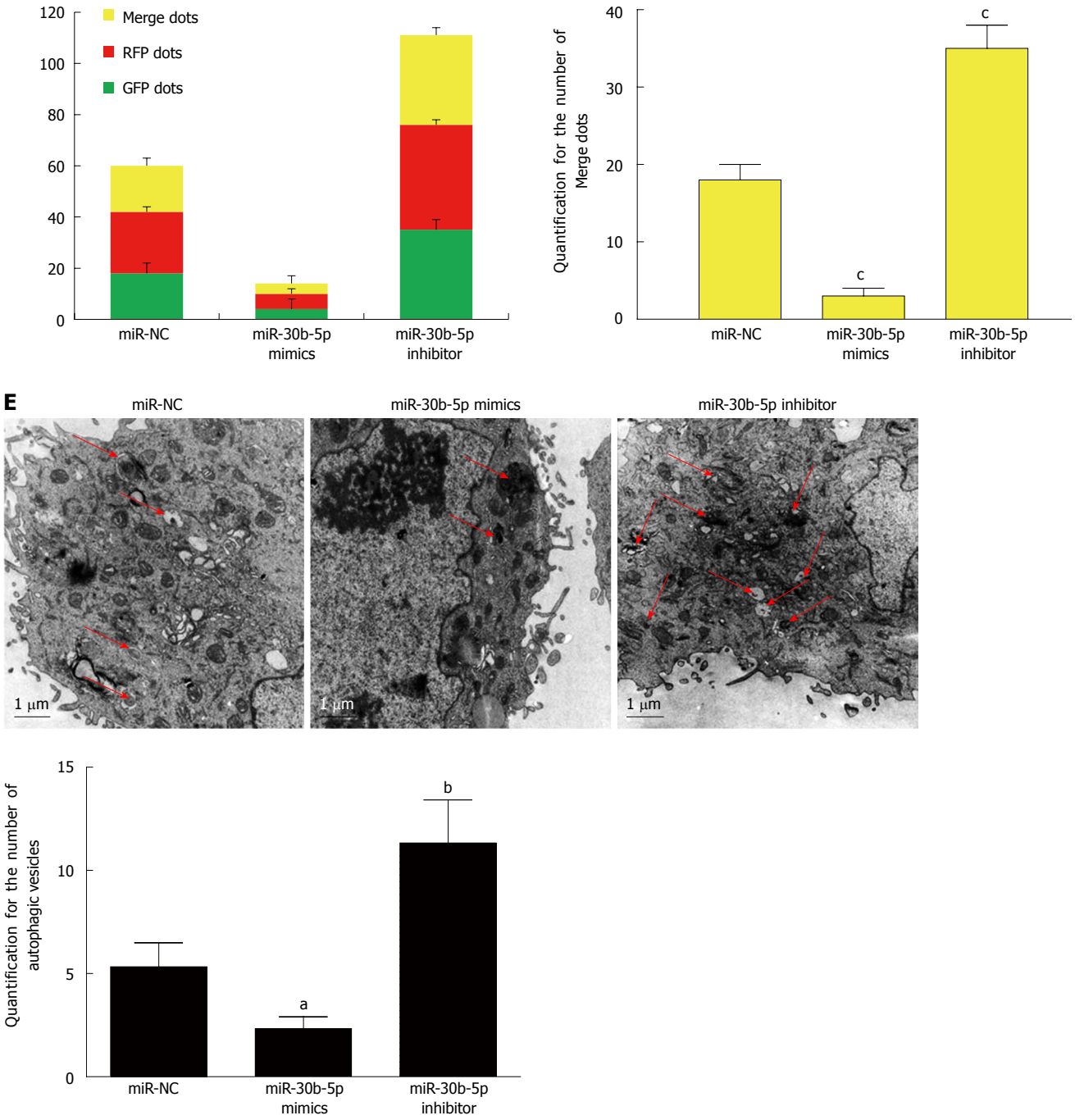

Figure 3 miR-30b inhibits autophagy by sequestering Atg12 in AML12 cells. A and B: The predicted miR-30b binding site on the Atg12 mRNA 3'-untranslated region (UTR) is shown, and a luciferase reporter assay was performed to determine the effects of miR-30b on the Atg12 mRNA 3'-UTR; C: Western blot was used to detect expression of Atg12 and LC3 in AML12 cells treated with miR-30b mimics or inhibitor; D: Confocal immunofluorescence of AML12 cells demonstrated increased numbers of GFP-RFP-LC3 dots in the IR group; when autophagy is induced, both GFP and RFP are expressed as yellow dots (autophagosomes). Red dots represent autolysosomes as the GFP degrades in an acid environment. Scale bars = 20 μm; E: TEM images of AML12 cells after ischemia followed by reperfusion at 12 h. TEM images show representative examples of autophagosomes (red arrows). Scale bars = 1.0 μm. And the data were quantified by counting the number of autophagosomes per cross-sectioned cell; $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* miR-NC group. Every experiment was repeated three times.

indicate that miR-30b could decrease Atg12 and Atg12-Atg5 conjugate levels in AML12 cells.

We performed a Ad-GFP-RFP-LC3 to examine the potential role of miR-30b in autophagy after IRI in AML12 cells. The appearance of GFP- or RFP-LC3 dots within the cytoplasm reflects the recruitment of LC3 proteins to autophagosomes. Upon activation of autophagy, both GFP and RFP are expressed as yellow dots when merged, representing autophagosomes. When autophagosomes fuse with lysosomes and form autolysosomes, the GFP degrades in an acid environment, but the RFP-LC3 remains showing as red dots. A confocal immunofluorescence experiment was performed to demonstrate an increase of LC3. The Ad-GFP-RFP-LC3 was transfected into AML12 cells to confirm the induction of autophagy. As shown in

Figure 3D, miR-30b inhibited significantly the induction of autophagosomes. We observed both fluorescent proteins were expressed after the infection with Ad-GFP-RFP-LC3. There were significant decreases in yellow dots with marginal elevations in red dots in the miR-30b-5p mimics group compared to the miR-NC or miR-30b-5p inhibitor groups ($P < 0.001$). Next, we evaluated autophagic vacuoles using TEM, an Autophagosomes were clearly visualized with TEM (Figure 3E). The basal number of autophagosomes within the miR-30b-5p mimics group was decreased relative to the miR-30b-5p inhibitor or miR-NC group ($P < 0.001$). Taken together, the results show that miR-30b inhibits autophagic flux by sequestering Atg12.

miR-30b inhibits autophagy to alleviate AML12 cell ischemia-reperfusion injury by targeting Atg12

In order to assess whether cells become more resistant to IRI depending on autophagy status in a hepatic IRI model, we examined the effects of rapamycin and 3-MA, an activator and inhibitor of autophagy, respectively. Rapamycin decreased the survival ratio of AML12 cells induced by IR treatment, and 3-MA inhibited this change ($P < 0.05$, Figure 4A). Moreover, rapamycin increased the levels of LC3Ⅱ and decreased P62 expression, whereas 3-MA decreased the levels of LC3Ⅱ and increased P62 expression (Figure 4B). These data found that activating autophagy could aggravate hepatic IRI. miR-30b-5p mimics increased viability of IRI AML12 cells treated responding to IR ($P < 0.05$), but viability of IRI AML12 cells treated with miR-30b-5p inhibitor was decreased ($P < 0.05$, Figure 4C). As demonstrated in Figure 4D, Atg12 siRNAs significantly down-regulated Atg12 expression in AML12 cells. The viability of IRI AML12 cells treated with miR-30b-5p mimics or inhibitor (Figure 4E) was enhanced by siRNA knockdown of Atg12. AML12 cells were also treated with miR-30b-5p mimics/inhibitor or Atg12 siRNA to investigate potential interactions between the miR-30b and Atg12-Atg5 conjugate during IR. At 12 h post-reperfusion, siRNA-mediated knockdown of Atg12 contributed to Atg12-Atg5 conjugate and LC3II protein levels inhibited by miR-30b-5p mimics, while Atg12 siRNA decreased the levels of Atg12-Atg5 conjugate and LC3II induced by miR-30b-5p inhibitor (Figure 4F). These data suggest that miR-30b inhibits autophagy to alleviate hepatic IRI by targeting Atg12.

DISCUSSION

Autophagy plays a pivotal role in cellular homeostasis and adaptation to adverse environments[12,13], and although the regulation of this process remains incompletely understood[14], it is known to provide a cytoprotective role important for survival[15]. Autophagy is regarded as a natural and essential defense mechanism against inflammatory, damnification, and oncotherapy[16]. Hence, regulation of the autophagy pathway has been implicated in the pathogenesis of numerous human diseases. Recently, a growing number of studies on autophagy and liver diseases have focused on liver ischemia reperfusion[17-20]. miR-30b, a member of the miR-30 family, has been suggested to play a role in the differentiation of several cell types[21]. The miR-30 family is also involved in the control of structural changes in the extracellular matrix of the myocardium[22] and in the regulation of the apoptosis[23].

In this study, we discovered that miR-30b was downregulated in mice livers subjected to IR. In addition, the expression of Atg12 and LC3II was upregulated, and the numbers of autophagosomes observed in the IR group increased as a function of time following reperfusion. miR-30b expression was decreased in response to hepatic IRI and was accompanied by a corresponding activation of autophagy. The induction of autophagy represents an initial response to IR in mice livers, while Atg12 and Atg12-Atg5 conjugate expression decreased as a function of time following reperfusion. To clarify whether miR-30b can alleviate hepatic IRI, we upregulated or downregulated expression of miR-30b in mice after tail intravenous injection of miR-30b-5p agomir or antagomir, respectively. miR-30b-5p agomir significantly decreased the histopathologic changes of livers induced by IR treatment, and the number of TUNEL-positive cells were significantly decreased. However, downregulated expression of miR-30b could aggravate the histopathologic changes. PCNA, a subunit of the mammalian DNA polymerase delta, is synthesized primarily during the S phase of the cell cycle[24]. PCNA is a relay molecule that functions as a molecular integrator for proteins involved in the control of the cell cycle, DNA repair, and cell death[25]. Therefore, PCNA is a convincing marker to distinguish proliferating cells. The miR-30b-5p agomir resulted in a significant increase in PCNA expression but a decrease in caspase-3, cleave caspase-3 and PARP1 expression. In contrast, down-regulated expression of miR-30b decreased in PCNA expression but increase in caspase-3, Cleave caspase-3 and PARP1 expression. These findings demonstrate that miR-30b can alleviate hepatic ischemia-reperfusion injury.

Autophagosome formation requires two ubiquitin-like conjugation systems, the Atg12 and LC3 systems[26]. The Atg12 system is located upstream of the LC3 system in the context of Atg protein organization. Atg12 is conjugated to Atg5, forming the irreversible Atg12-Atg5 complex, which strongly enhances the formation of LC3-phosphatidylethanolamine conjugation[27]. caveolin-1 also regulated Atg12-Atg5 conjugate during autophagosome formation, and caveolin-1 competitively interacts with the Atg12-Atg5 system to suppress the formation and function of the latter in lung epithelial cells[28]. Based upon miRNA target gene prediction, we identified the 3′-UTR area of the Atg12 gene as a match of miR-30b.

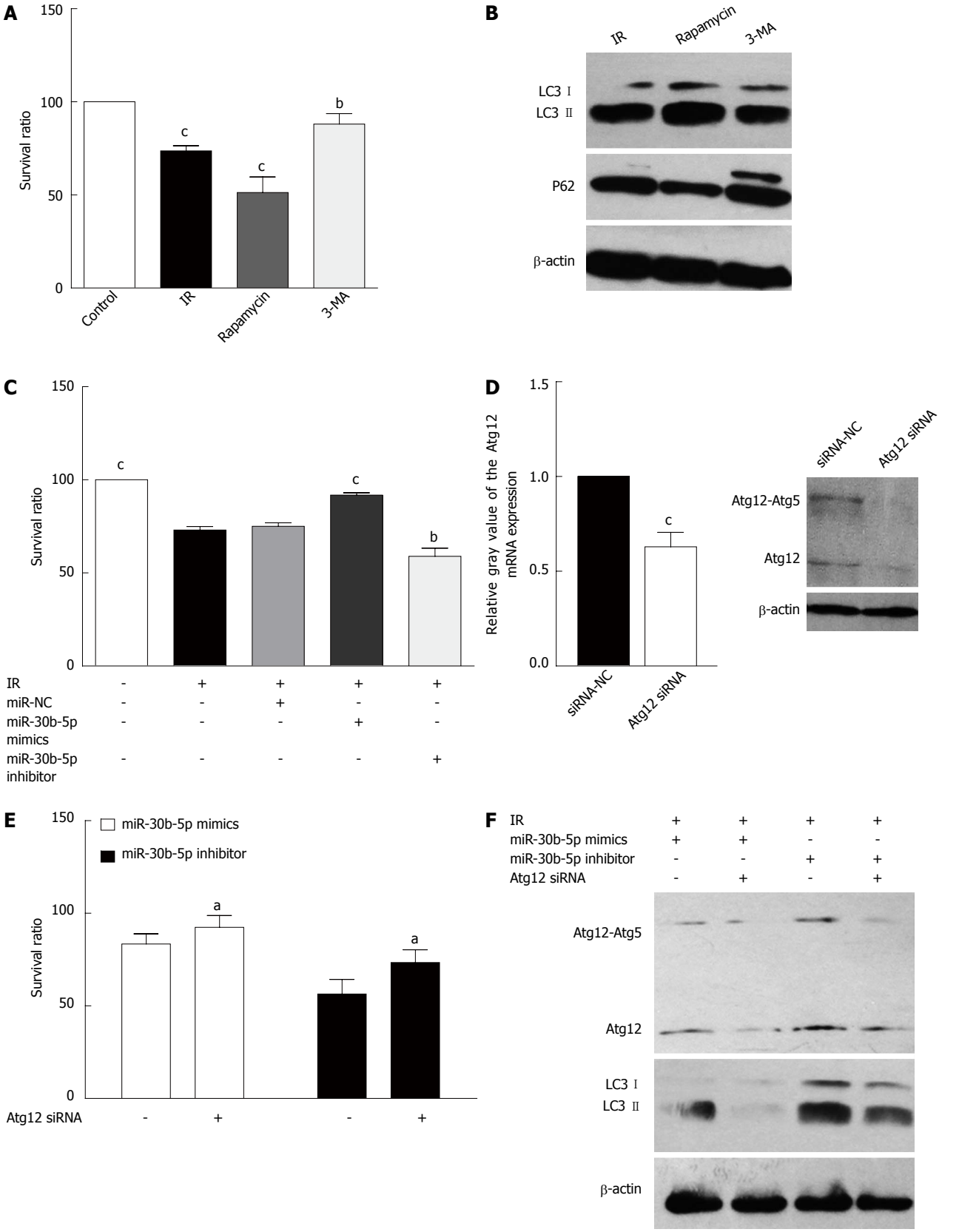

Figure 4 miR-30b alleviate AML12 cell ischemia-reperfusion injury by targeting Atg12 *in vitro*. A: The survival ratio of AML12 cells was measured after treatment with rapamycin or 3-MA; B: Western blot was used to detect the expression of LC3 and P62 in AML12 cells treated with Rapamycin or 3-MA; C: The survival ratio of AML12 cells was measured after treatment with miR-30b mimics or inhibitor; D: AML12 cells were transfected with Atg12 siRNAs, and the Atg12 mRNA and protein level of the target were evaluated using quantitative real time polymerase chain reaction or western blot analysis, respectively; E: The survival ratio of AML12 cells was measured at 12 h post-reperfusion in the absence or presence of miR-30b mimics, the miR-30b inhibitor, or Atg12 siRNA; F: Protein levels of Atg12 and LC3 were analyzed using western blot in AML12 cells at 12 h post-reperfusion in the absence or presence of miR-30b mimics, the miR-30b inhibitor, or Atg12 siRNA; $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* IR/miR-NC group. Every experiment was repeated three times.

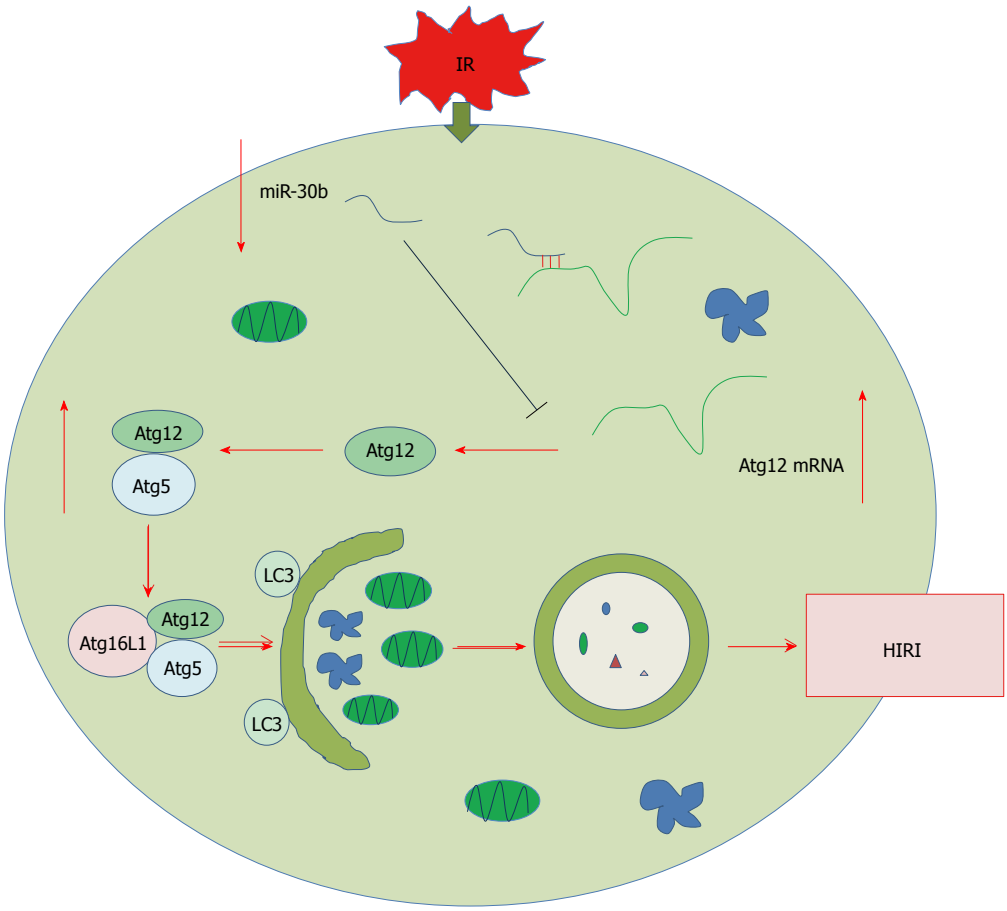

Figure 5 Model of miR-30b inhibition of autophagy to alleviate hepatic ischemia-reperfusion injury *via* decreasing the Atg12-Atg5 conjugate. The expression of miR-30b, which binds the 3'-UTR of Atg12, was significantly down-regulated after HIRI. Levels of Atg12 and Atg12-Atg5 conjugate then increased and promoted autophagy, leading to apoptotic cell death. Therefore, miR-30b can inhibit autophagy to alleviate hepatic IRI by decreasing levels of Atg12-Atg5 conjugate. HIRI: Hepatic ischemia-reperfusion injury.

Our data showed that up-regulation of the expression of miR-30b significantly increased cell viability as a function of time following reperfusion, while the miR-30b inhibitor significantly decreased cell viability. Related to these findings, a significant decrease of LC3 dots in AML12 transfected with miR-30b-5p mimics compared with miR-NC group. The number of LC3 dots increased in the miR-30b inhibitor group relative to the miR-30b mimics group. These results indicate that miR-30b increased the viability of hepatocytes induced by IR *via* inhibiting autophagy. Over-expression of miR-30b significantly reduced Atg12 and Atg12-Atg5 conjugate protein levels after AML12 cells transfected with miR-30b mimics; and LC3II expression was decreased in these cells. The expression of Atg12 and Atg12-Atg5 conjugate protein levels increased after AML12 cells were transfected with the miR-30b inhibitor, while LC3II was also increased at the same time. Taken together, it seems clear that miR-30b can decrease Atg12 and Atg12-Atg5 conjugate expression, thereby down-regulating autophagy to alleviate hepatic IRI.

Autophagy is a self-digesting process that occurs in response to stress and plays important roles in the pathogenesis of a variety of diseases[15]. Autophagy functions mainly in a pro-survival capacity for cells to cope with nutrient starvation and anoxia[29,30], however, excessive levels of autophagy within impaired cells can contrarily induce cell death[31]. Oxidative stress may lead to autophagy which induces cell death in cisplatin-induced AKI[32]. In our study, we found that activation of autophagy aggravated hepatic IRI, an effect that was dependent on Atg12 and Atg12-Atg5 conjugate. We also treated AML12 cells with the miR-30b mimics, miR-30b inhibitor, or Atg12 siRNA to investigate whether any potential interaction may exist between miR-30b and Atg12 during IR. Our findings suggest that miR-30b mediated apoptosis to alleviate hepatic IRI, an effect that was dependent on Atg12 activity. As was shown in Figure 5, we found that miR-30b inhibited autophagy to alleviate hepatic IRI by decreasing the Atg12-Atg5 conjugate. This finding may serve as a guide to prevent hepatic IRI and provide a future strategy in research areas of IRI.

ACKNOWLEDGMENTS

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Center of Tianjin First Central Hospital for their technical support.

COMMENTS

Background

Hepatic ischemia reperfusion injury (IRI) represents an important clinical problem as related to liver resection or transplantation. miRNAs participate in various hepatic pathophysiological processes *via* regulating autophagy. miR-30b, a member of the miR-30 family, is involved in the control of structural changes in the regulation of the apoptosis. However, the importance and function of miR-30b and whether miR-30b regulate autophagy to alleviate hepatic IRI have not yet been elucidated.

Research frontiers

It was reported that overexpression of miR-30b had an anti-apoptotic effect on the early phase of rat myocardial ischemia injury model through targeting Kirsten ras sarcoma (KRAS) and activating the Ras/Akt pathway. E2F1-regulated miR-30b suppressed Cyclophilin D to protect the heart from IRI and necrotic cell death.

Innovations and breakthroughs

This is the first study to investigate the role of miR-30b in inhibiting autophagy to alleviate hepatic IRI, and we found that miR-30b decreases the level of Atg12-Atg5 conjugate. This study provides the basis for a future research strategy in hepatic ischemia reperfusion injury.

Applications

This study provides insight into the role of miR-30b in the inhibition of autophagy to alleviate hepatic IRI by decreasing the autophagy-related gene Atg12-Atg5 conjugate. Understanding how miR-30b regulates autophagy during hepatic IRI may facilitate the design of new therapeutic approaches to prevent and cure hepatic IRI.

Terminology

Atg12 ubiquitin-like conjugation systems are required in autophagosome formation, and the Atg12 system is located upstream of the light chain 3 (LC3) system in the context of Atg protein organization. Atg12 is conjugated to Atg5, forming the irreversible Atg12-Atg5 complex, which strongly enhances the formation of LC3-phosphatidylethanolamine conjugation.

Peer-review

This is an interesting paper, providing important information on the expression of miR-30b and its role in autophagy during hepatic IRI. This study found that the miR-30b inhibited autophagy to alleviate hepatic IRI *via* decreasing the Atg12-Atg5 conjugate. The result is very important for advanced research studies on hepatic IRI.

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ORIGINAL ARTICLE

Basic Study

Dissecting characteristics and dynamics of differentially expressed proteins during multistage carcinogenesis of human colorectal cancer

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Abstract

AIM: To discover novel biomarkers for early diagnosis, prognosis or treatment of human colorectal cancer.

METHODS: iTRAQ 2D LC-MS/MS analysis was used to identify differentially expressed proteins (DEPs) in

the human colonic epithelial carcinogenic process using laser capture microdissection-purified colonic epithelial cells from normal colon, adenoma, carcinoma *in situ* and invasive carcinoma tissues.

RESULTS: A total of 326 DEPs were identified, and four DEPs (DMBT1, S100A9, Galectin-10, and S100A8) with progressive alteration in the carcinogenic process were further validated by immunohistochemistry. The DEPs were involved in multiple biological processes including cell cycle, cell adhesion, translation, mRNA processing, and protein synthesis. Some of the DEPs involved in cellular process such as "translation" and "mRNA splicing" were progressively up-regulated, while some DEPs involved in other processes such as "metabolism" and "cell response to stress" was progressively down-regulated. Other proteins with up- or down-regulation at certain stages of carcinogenesis may play various roles at different stages of the colorectal carcinogenic process.

CONCLUSION: These findings give insights into our understanding of the mechanisms of colorectal carcinogenesis and provide clues for further investigation of carcinogenesis and identification of biomarkers.

Key words: Colorectal Cancer; Proteome; Biomarker; Carcinogenesis

Core tip: In this study, we used iTRAQ 2D LC-MS/MS analysis to identify differentially expressed proteins (DEPs) in the human colonic epithelial carcinogenic process using laser capture microdissection-purified colonic epithelial cells from normal colon, adenoma, carcinoma *in situ* and invasive carcinoma tissues. A total of 326 DEPs were identified. Four DEPs (DMBT1, S100A9, Galectin-10, and S100A8) with progressive alteration in the carcinogenic process were further validated using immunohistochemistry. The DEPs were involved in multiple biological processes including cell cycle, cell adhesion, translation, mRNA processing, and protein synthesis. These findings give insights into our understanding of the mechanisms of colorectal carcinogenesis and provide clues for further investigation of carcinogenesis and identification of biomarkers.

Peng F, Huang Y, Li MY, Li GQ, Huang HC, Guan R, Chen ZC, Liang SP, Chen YH. Dissecting characteristics and dynamics of differentially expressed proteins during multistage carcinogenesis of human colorectal cancer. *World J Gastroenterol* 2016; 22(18): 4515-4528 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i18/4515.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i18.4515

INTRODUCTION

Colorectal cancer (CRC) is the third leading cause of cancer death, affecting over a million people worldwide per year in recent years[1,2]. The World Health Organization estimates a 77% increase in the number of newly diagnosed cases of CRC and an 80% increase in deaths from CRC by 2030[3]. Despite an improvement in relative survival of CRC at 5 years due to early diagnosis at initial stages and breakthroughs in treatment of stages Ⅱ and Ⅲ disease, CRC is still one of the most lethal malignancies and the 5-year survival rate for patients with metastasis is < 5%[4]. Most patients are diagnosed at an advanced stage and have a poor prognosis. At present, CRC diagnosis and therapy are still dependent upon descriptive classification and staging systems based on morphology/histology[5]. Remarkable achievements in the understanding of cellular and molecular mechanisms of colorectal carcinogenesis have been made in recent years. However, therapy for advanced colorectal cancer remains limited, and current screening methods including sigmoidoscopy and colonoscopy lack the required sensitivity and specificity[6,7]. Therefore, a comprehensive understanding of the mechanisms behind colorectal carcinogenesis will contribute to the improvement in early detection and prognosis and provide novel therapeutic targets.

Carcinogenesis is a multistep and complicated process characterized by genetic alterations, including chromosomal abnormalities, gene mutations, and epigenetic changes that disrupt normal cell growth and division[8]. A genetic model describing the transition from healthy colonic epithelia through dysplastic adenoma to malignant cancer has been proposed[9]. According to the model[10], the colonic carcinogenic process originates from normal colonic mucosa (NC) and then transforms sequentially from adenoma (AD), carcinoma *in situ* (CIS, equivalent to high-grade intraepithelial neoplasia), and ultimately to invasive colorectal cancer (ICC)[11]. A number of sequential genetic abnormalities, including gene mutations in APC, K-ras, and p53 and epigenetic changes, were identified at different stages of colorectal carcinogenesis[11,12].

In recent years, much progress has been made in understanding genetic changes in the colonic carcinogenesis process, and many studies have been conducted to analyze differentially expressed proteins (DEPs) between certain stages of colorectal carcinogenesis[13-15]. However, there has been no systematic comparison between typical stages across the carcinogenic process, and much less is known about dynamic alterations at the proteome level during the process. Tissue heterogeneity is the main problem for analysis of biological samples in the study of disease. Recent technological progress using laser

capture microdissection (LCM) has made it possible to overcome this problem and to enrich the desired populations of cells from heterogeneous tissues[16-18].

Isobaric tags for relative and absolute quantification (iTRAQ) combined with two-dimensional liquid chromatography-tandem mass spectrometry (2D LC-MS/MS) is a highly sensitive and practical technology[19-21]. Compared to conventional proteomic technology such as 2D electrophoresis, the iTRAQ method has the following advantages. First, it can label proteins from up to eight samples in a single experiment. Second, it can resolve large proteins (> 200 kD), small proteins (< 10 kD), and proteins with extremes in isoelectric point[22]. Therefore, iTRAQ technology offered us a feasible method to simultaneously compare the proteomes of successive stages of colorectal carcinogenesis.

To clarify the dynamic patterns of DEPs during colorectal carcinogenesis and provide valuable information for further identification of biomarkers for prevention, treatment or early diagnosis of CRC, iTRAQ tagging followed by 2D LC-MS/MS was performed to identify DEPs among LCM-purified colonic epithelial carcinogenic tissues and explore their dynamic expression patterns. A total of 326 DEPs were identified among different stages to have distinct expression patterns during the carcinogenic process, and four top-ranked DEPs (DMBT1, S100A9, Galectin-10, and S100A8) were further validated by immunohistochemistry. To our knowledge, this is the first comprehensive study that systematically compares the dynamic alterations of proteins during the process of colorectal carcinogenesis by comparative proteomics.

MATERIALS AND METHODS

Sample collection

Twenty-seven cases of fresh colonic tissues (5 cases of NC, 8 cases of AD, 5 cases of CIS, 9 cases of ICC) collected between January 2011 and December 2012 were obtained from the Department of Surgery, Xiangya Hospital, Central South University, China and used for iTRAQ-labelling. The patients received neither chemotherapy nor radiotherapy before curative surgery and signed an informed consent form for the study, which was approved by the local ethical committee. All tissue specimens were obtained from surgical resection, and the normal colonic tissue samples were acquired from the resection edge furthest away from the lesion (≥ 10 cm). The tissue samples in the ICC group were from CRC patients with lymph node metastasis. All of the tissues were flash frozen in liquid nitrogen and stored at -80 ℃ until further use.

An independent set of formalin-fixed and paraffin-embedded archival tissue specimens, including 50 cases of NC, 50 cases of AD, 30 cases of CIS, and 63 cases of ICC, were obtained from colonoscopic or surgical resection at Xiangya Hospital, Central South University, China, and used for immunohistochemical staining. The parameters of patients and tissue specimens are shown in Supplementary Table 1.

Tissue processing and LCM

To exclude the interference of stromal elements and adjoining cells, LCM[18] was used to purify the target cells. All NC, AD, CIS and ICC tissues were stained with haematoxylin and eosin and independently evaluated by two experienced pathologists. LCM was performed to purify the cells of interest from each type of tissue according to our previous procedure[17]. Briefly, frozen sections (8 μm thick) of all tissues were prepared using a Leica CM 1900 cryostat at -25 ℃. The sections were placed on membrane-coated glass slides (Leica), fixed in 75% alcohol for 30 s, and stained with 0.5% violet-free methyl green (Sigma). The stained sections were air-dried and then subjected to LCM. Each cell population was determined to be 95% homogeneous by microscopic visualization of the captured cells (Supplementary Figure 1).

Protein extraction

The microdissected cells were dissolved in lysis buffer (7 mol/L urea, 2 mol/L thiourea, 65 mmol/L dithiothreitol, 0.1 mmol/L phenylmethylsulfonyl fluoride) at 4 ℃ for 1 h and then centrifuged at 12000 rpm for 30 min at 4 ℃. The supernatant was collected, and the protein concentration was determined by the 2D Quantification Kit (Amersham Biosciences). To diminish the effects of biological variation on the proteomic results, equal amounts of proteins from each individual sample in various types of tissue (NC, AD, CIS and ICC) were pooled to generate a sample for the corresponding type of tissue. Four pooled protein samples (corresponding to the four types of tissues) were obtained for iTRAQ labelling.

Protein digestion and labelling with iTRAQ reagents

Trypsin digestion and iTRAQ labelling were performed according to the manufacturer's protocol (Applied Biosystems). In brief, 100 μg protein of each pooled sample was reduced, alkylated, and then digested overnight at 37 ℃ with trypsin (mass spectrometry grade; Promega). The samples were then labelled with iTRAQ reagents as follows: iTRAQ reagent 113, AD; iTRAQ reagent 114, NC; iTRAQ reagent 115, CIS; and iTRAQ reagent 116, ICC. The labelled digests were then mixed and dried.

Off-line 2D LC-MS/MS

The mixed peptides were fractionated according to the procedure described in our previous study[23]. A total of 10 SCX fractions were collected. Each fraction was dried down by the rotary vacuum concentrator, dissolved in buffer C (5% acetonitrile, 0.1% formic acid), and analysed on Triple TOF 5600 systems (Applied Biosystems) in information dependent mode. Briefly, peptides were separated on reverse-phase

columns (ZORBAX 300SB-C18 column, 5 μm, 300 Å, 0.1 mm × 15 mm; Micromass) using an Eksigent 1D PLUS system (Applied Biosystems). Peptides were separated by a linear gradient mobile phase A (5% acetonitrile, 0.1% formic acid) and mobile phase B (95% acetonitrile, 0.1% formic acid) from 5 to 40 of mobile phase B in 120 min at a flow rate of 300 nL/min. Survey scans were acquired from 400-1500 with up to 15 precursors selected for MS/MS and dynamic exclusion for 20 s. Each SCX fraction was analysed in duplicate.

Data analysis

Analyst QS 1.1 (Applied Biosystems) was used for data acquisition, and ProteinPilot 4.2 (Applied Biosystems) was used for protein identification and quantification. The precursor tolerance and the iTRAQ fragment tolerance were both set at 0.2 Da. The data analysis parameters were set as follows: Sample type, Itraq 4 plex (peptide labelled); Cys alkylation, MMTS; Digestion, Trypsin; Instrument, Triple TOF 5600; Species, Homo sapiens; ID Focus, Biological modifications; Database, Uniprot human database (release Apr 2013); Search Effort, Thorough; Max missed cleavages, 2; FDR Analysis, Yes; User Modified Parameter Files, No; Bias Correction, Auto; Background Correction, Yes. Identified proteins were grouped by the software to minimize redundancy. All peptides used for the calculation of protein ratios were unique to the given protein or proteins within the group, and peptides that were common to other isoforms or proteins of the same family were ignored.

The protein confidence threshold cutoff was 1.3 (unused ProtScore) with at least one peptide with 95% confidence. The average iTRAQ ratios from the duplicate experiments were calculated for each protein[23,24]. The confidence level of the altered expression of proteins was calculated by ProteinPilot as a *P*-value, which allows the results to be evaluated based on the confidence level of expression change. In addition, false discovery rate (FDR) for the protein identification was calculated by searching against a concatenated reversed database[25].

Determination of cutoff threshold for significant fold changes in iTRAQ experiments

In a comprehensive iTRAQ experiment, the variations are composed of technical, experimental and biological variations[26]. According to previous reports[26-28], the cutoff threshold for meaningful fold changes over experimental errors can be determined by using the experimental replicate method. Particularly, the main variation in this study was experimental variation, whereas the biological variation was minimized by sample pooling effect. The variations in our method using experimental replicates are considered to be the actual variations in the iTRAQ experiment.

The number of shared quantified proteins in the 2 iTRAQ experiments was 379 based on the following selection criteria: containing more than 2 unique peptides (> 95%), *P*-value < 0.05, and EF < 2. These 379 proteins were used initially to determine the experimental variations and to confirm the threshold for meaningful fold changes.

Experimental variations for the 113/114, 115/114, and 116/114 reporter ions were calculated using the ratios of the 379 common quantified proteins between the first and second iTRAQ experiments; the experimental variations for the reporter ions were r^2 = 0.8499, r^2 = 0.8382, and r^2 = 0.9283, respectively (Supplementary Figure 2A). In addition, the cutoff threshold for meaningful fold changes (cutoff for DEPs) in the expression ratios of 113/114, 115/114, and 116/114 were determined using the experimental replicate method described in previous studies[27,28]. Accordingly, 90% of the identified proteins in the 2 iTRAQ experiments fell within 50% of the respective experimental variation (Supplementary Figure 2B). Therefore, only fold changes > 1.5 or < 0.67 were considered significant.

Differential proteome expression during colorectal carcinogenesis

To identify the DEPs in the colorectal carcinogenic process, protein expression profiles between two stages of this process (AD, CIS or ICC *vs* NC; CIS *vs* AD; ICC *vs* CIS) were compared. The proteins that met the following criteria were confidently considered as DEPs: (1) proteins were repeatedly identified by the two experiments; (2) proteins were identified based on ⩾ 2 peptides; (3) an averaged ratio-fold change > 1.5 or < 0.67 between two stages; (4) proteins in CIS or ICC should be differentially expressed compared with NC; and (5) proteins should be differentially expressed in at least one of the three comparisons between adjacent stages (AD *vs* NC, CIS *vs* AD and ICC *vs* CIS).

Cluster analysis of differential protein expression profiles

The averaged iTRAQ values obtained in two experimental replicates for each protein of the four types of tissues (NC, AD, CIS, and ICC) were log2-transformed. Total DEPs were normalized and clustered with J-express 2012 (http://jexpress.bioinfo.no) into nine categories by using the K-means algorithm with the Pearson correlation distance.

Bioinformatics analysis

The DEPs were first annotated by GO from Biological Process using David software (http://david.abcc.ncifcrf.gov/). The GO terms were considered to be significantly enriched when the corrected *P*-value was less than 0.05. Pathway analyses of each K-means cluster of proteins were performed using REACTOME software (http://www.reactome.org/). The REACTOME

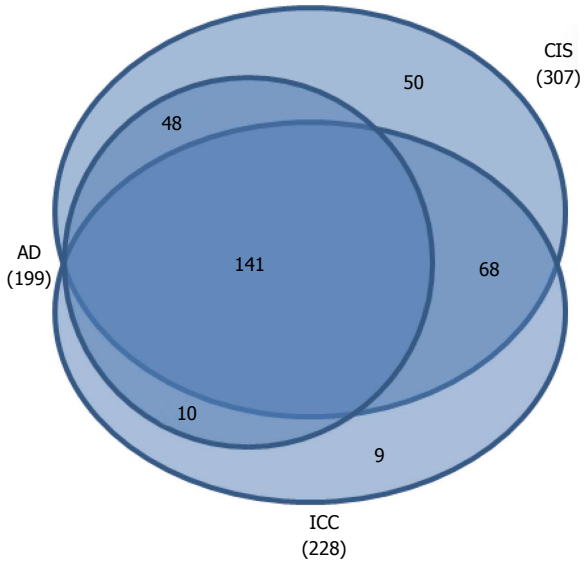


Figure 1 Venn diagrams of comparisons of the differentially expressed proteins from the different stages compared to the NC group.

performs an enrichment test to determine whether any Reactome pathways are enriched in the submitted data. A binomial test was used to calculate the probability. The *P*-values are corrected for multiple testing (Benjamini-Hochberg procedure) that arises from evaluating the submitted list of identifiers against every pathway. The pathway with the corrected *P*-value less than 0.05 was considered to be significantly enriched.

Immunohistochemistry and evaluation of staining

Immunohistochemistry was performed according to the procedure described in our previous study[26]. Briefly, the sections were incubated with anti-DMBT1 (1:200; Santa Cruz), anti-S100A9 (1:200; Santa Cruz), anti-Galectin-10 (1:200; Abcam), or anti-S100A8 (1:200; Santa Cruz) antibody overnight at 4 ℃, and they were then incubated with a biotinylated secondary antibody followed by avidin-biotin peroxidase complex (DAKO) according to the manufacturer's instructions. Finally, tissue sections were incubated with 3',3'-diaminobenzidine until a brown colour developed and were then counterstained with Harris' modified haematoxylin. The evaluation of immunostaining was performed as previously described[29]. A score (ranging from 0-6) was obtained for each case. A combined staining score of $\leqslant$ 2 was considered to be negative staining (no expression); a score between 3 and 4 was considered to be moderate staining (expression); and a score between 5 and 6 was considered to be strong staining (high expression).

Statistical analysis

SPSS software (IBM, v19) was used for statistical analyses. Numerical variables with normal distribution were compared using unpaired *t*-tests or paired *t*-tests. Non-normal distribution data were compared using Wilcoxon rank sum tests. A *P*-value less than 0.05 was considered statistically significant.

Table 1 Number of protein expression changes in comparison between adjacent stages of colon carcinogenesis process

Comparison	Protein expression	
	Downregulated	Upregulated
AD:NC (113:114)	113	86
CIS:AD (115:113)	137	102
ICC:CIS (116:115)	103	141

RESULTS

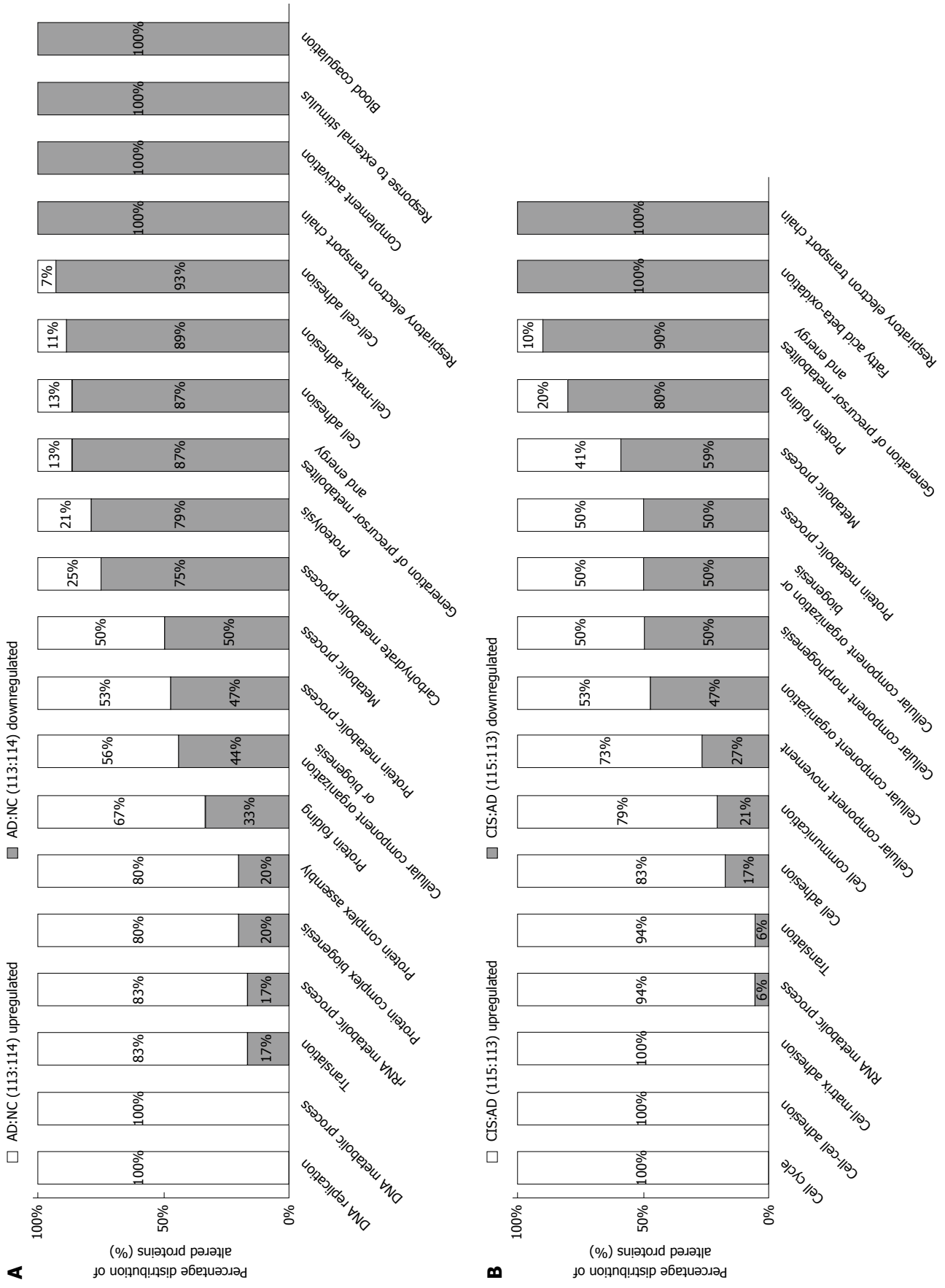
Identification of quantified proteins during colorectal carcinogenesis

A total of 3211 non-redundant proteins were identified at a minimum confidence level of 95% (unused ProtScore > 1.3) in two iTRAQ experiments. Among these, 2374 proteins were repeatedly identified in the two experiments. In total, 3123 proteins were quantified, and 2319 of them were commonly quantified in the two iTRAQ experiments (Supplementary Figure 3). The detailed protein identification and quantification data are shown in Supplementary Table 2. Using the concatenated target-decoy database search strategy as detailed by Elias and Gygi[25], a 0% rate of false positives was estimated, which further strengthened the reliability of our data.

To define significant changes in protein expression, fold-changes > 1.5 or < 0.66 were established as cutoff values, which were determined using the experimental replicate method as described in the Methods section. A total of 326 DEPs were found according to the selection criteria, of which 199 were found in AD/NC, 307 in CIS/NC, and 228 were in ICC/NC. There were 141 (43%) common DEPs among all three tumor stages compared to NC. All the DEPs in AD/NC group were found in CIS/NC or ICC/NC groups, while there were 50 DEPs specific to CIS/NC, and 9 DEPs specific to ICC/NC (Figure 1). Out of 326 DEPs, 199 were found in AD/NC (86 upregulated and 113 downregulated), 239 were found in CIS/AD (102 upregulated and 137 downregulated), and 244 in ICC/CIS (141 upregulated and 103 downregulated) (Table 1).

To obtain a biological view of the DEPs, GO enrichment analysis was employed to discover the significant biological processes. In the comparison of proteome expression in AD (113 reporter) *vs* NC (114 reporter), out of 199 DEPs, 86 up-regulated proteins were involved in gene expression processes such as "DNA replication", "DNA metabolic process" and "translation", whereas 113 down-regulated proteins were involved in energy and metabolism processes such as "blood coagulation", "response to external stimulus" and "complement activation" (Figure 2A).

Upon comparison of the proteomes of CIS (115


A
AD:NC (113:114) upregulated
AD:NC (113:114) downregulated
Percentage distribution of altered proteins (%)
100%
50%
0%
DNA replication
DNA metabolic process
Translation
RNA metabolic process
Protein complex biogenesis
Protein complex assembly
Protein folding
Cellular component organization or biogenesis
Protein metabolic process
Metabolic process
Carbohydrate metabolic process
Proteolysis
Generation of precursor metabolites and energy
Cell adhesion
Cell-matrix adhesion
Cell-cell adhesion
Respiratory electron transport chain
Complement activation
Response to external stimulus
Blood coagulation
B
CIS:AD (115:113) upregulated
CIS:AD (115:113) downregulated
Percentage distribution of altered proteins (%)
100%
50%
0%
Cell cycle
Cell-cell adhesion
Cell-matrix adhesion
RNA metabolic process
Translation
Cell adhesion
Cell communication
Cellular component movement
Cellular component morphogenesis
Cellular component organization or biogenesis
Protein metabolic process
Metabolic process
Protein folding
Generation of precursor metabolites and energy
Fatty acid beta-oxidation
Respiratory electron transport chain

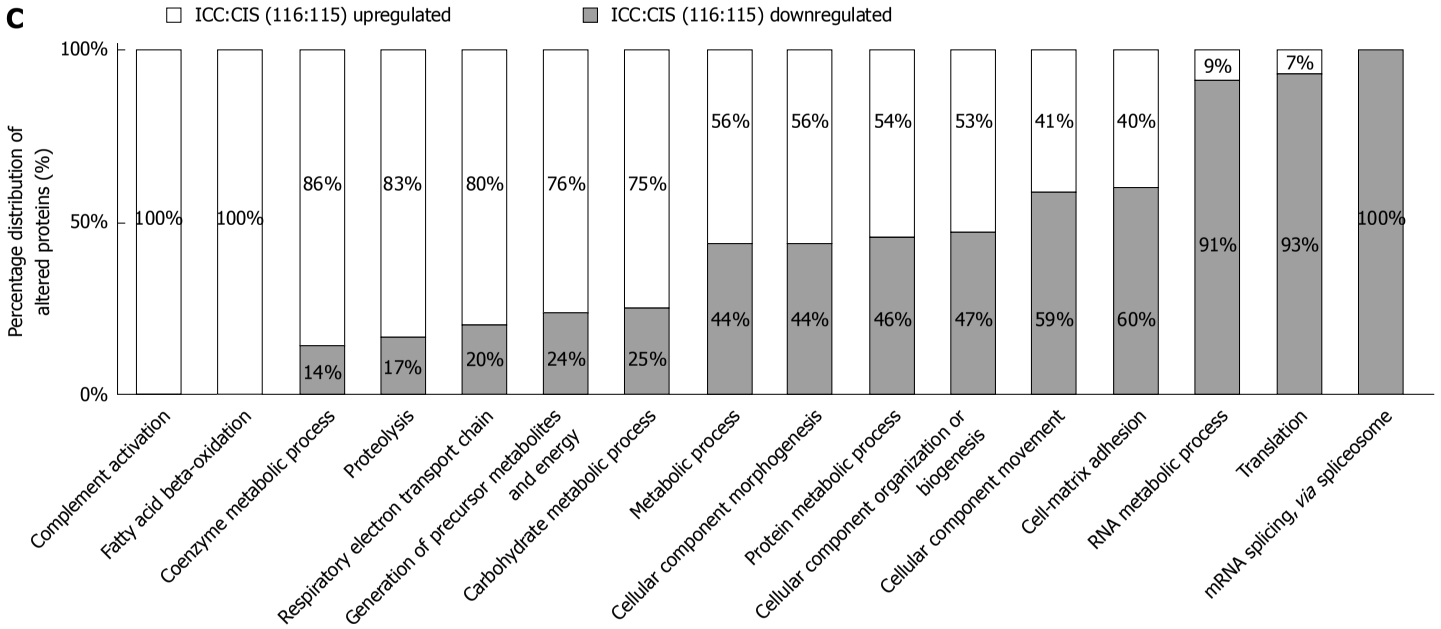


Figure 2 Functional distribution of differentially expressed proteins between adjacent stages of the colon carcinogenesis process. Functional classification of differentially expressed proteins from AD *vs* NC, CIS *vs* AD and ICC *vs* CIS were assigned to "biological process" subcategories. Only significant subcategories for "biological process" are presented. Each subcategory is presented as the percentage of up- and down-regulated proteins.

reporter) *vs* AD (113 reporter), we identified 239 DEPs, 102 and 137 of which were up-regulated and down-regulated, respectively. Notably, the down-regulated proteins were involved in energy metabolism pathways such as "respiratory electron transport chain", "fatty acid beta-oxidation" and "generation of precursor metabolites and energy", whereas the up-regulated proteins were involved in biological process such as "cell cycle", "cell-cell adhesion" and "cell-matrix adhesion" (Figure 2B).

Between the expression patterns of ICC (116 reporter) *vs* CIS (115 reporter), 244 DEPs included 141 up-regulated and 103 down-regulated proteins. The bioinformatics analysis indicated that the up-regulated proteins mainly participate in "complement activation", "fatty acid beta-oxidation" and "coenzyme metabolic processes", whereas the down-regulated proteins participate in biological processes associated with "mRNA splicing", "translation" and "cell-matrix adhesion" (Figure 2C).

Cluster analysis of DEPs and functional analysis

Among the DEPs, some were only differentially expressed in one stage, while others were differentially expressed in multiple stages. To explore the dynamics of DEPs and gain more insights into their biological significance in colorectal carcinogenic processes, K-means clustering and REACTOME pathway analysis were performed. The expression pattern K-means clustering analysis of DEPs showed stage-specific and co-regulated expression profiles.

The 326 DEPs were classified into 9 clusters by the K-means clustering algorithm (Figure 3, Supplementary Table 3). According to the overall tendency, the nine clusters were arbitrarily categorized into three groups. Group 1 consists of clusters 1 and 5, in which the abundance of DEPs increased at all three stages of AD, CIS and IC, and exhibited the highest expression in CIS or IC. Group 2 consists of cluster 2 and 7, in which the abundance of DEPs decreased at all three stages of AD, CIS

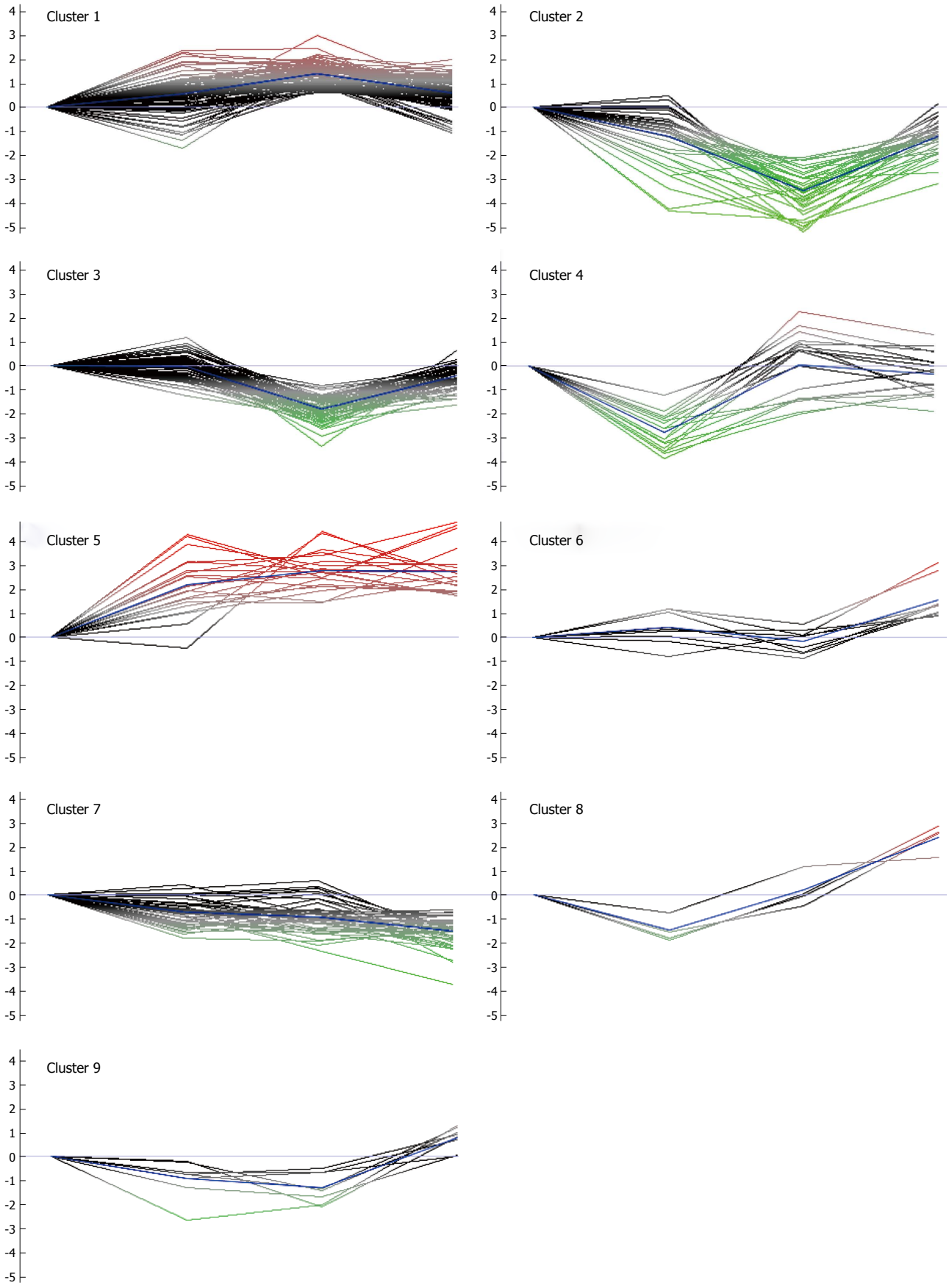


Figure 3 K-mean clusters of differentially expressed proteins. These proteins could be clustered into nine clusters. According to the average tendency, the nine clusters can be arbitrarily categorized into three groups. Group 1 includes clusters 1 and 5, in which the abundance of proteins progressively increased during the colon carcinogenic process. Group 2 consists of clusters 2 and 7, in which the abundance of proteins progressively decreased during the process. Group 3 consists of clusters 3, 4, 6, 8 and 9, in which the abundance of proteins was significantly up-regulated or down-regulated in certain stages of the process.

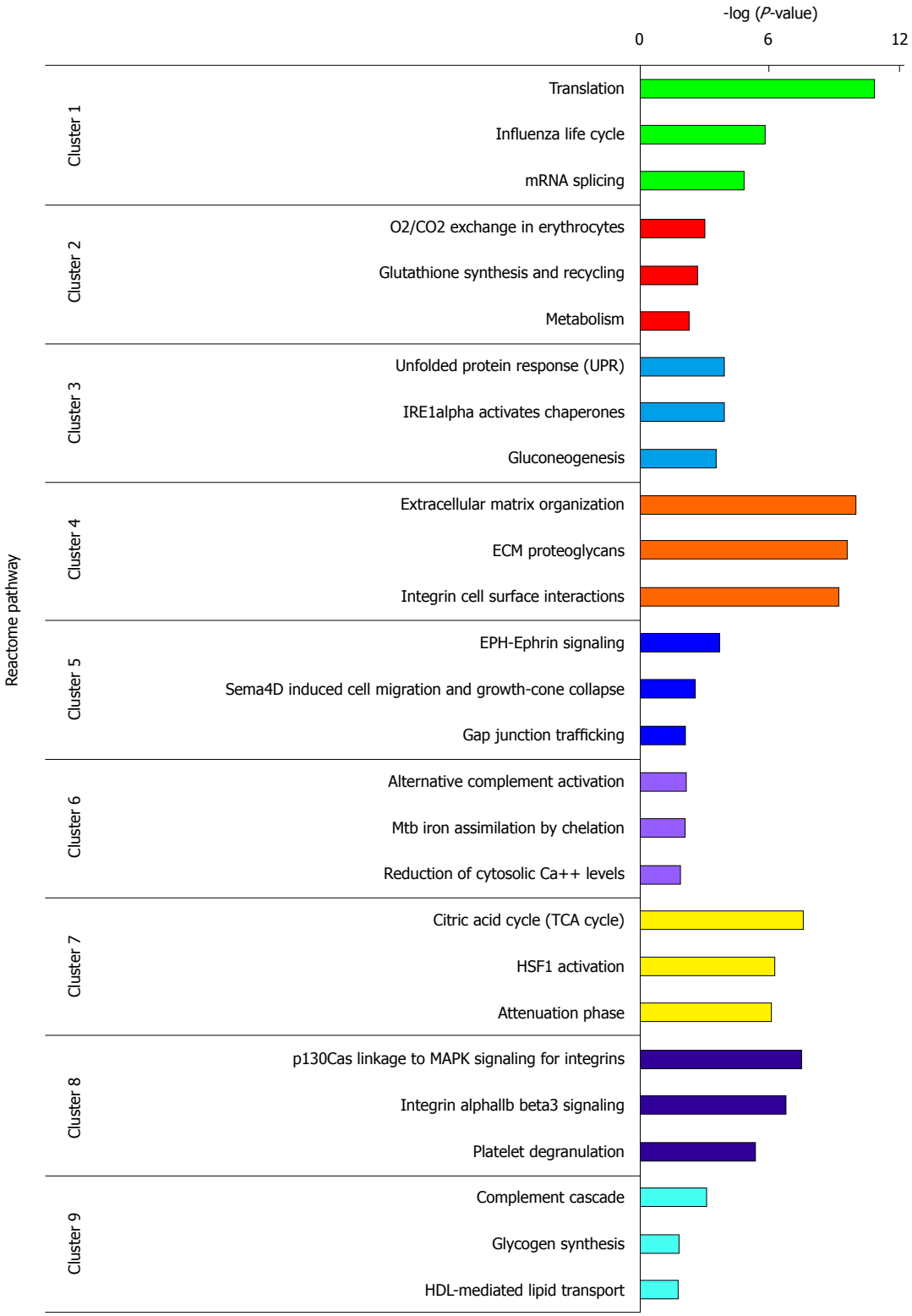


Figure 4 REACTOME pathway analysis of differential expressed proteins in each cluster. Only top 3 significant pathways are presented. Each pathway is presented as negative logarithm of *P*-value.

and IC, and exhibited the lowest expression in CIS or IC. Group 3 consists of clusters 3, 4, 6, 8 and 9, in which the abundance of proteins fluctuated during the colorectal carcinogenic process and was significantly up-regulated or down-regulated only in certain stages. Ideally, the proteins within each cluster are co-regulated proteins, and may have similar biological functions during colorectal carcinogenesis. Pathway analysis with REACTOME revealed that proteins in clusters 1 and 5 were mainly involved in "translation", "EPH-Ephrin signaling" and "Sema4D induced cell migration and growth-cone collapse", *etc*., whereas

Table 2 Top 10 differential proteins (up-regulated and down-regulated) between different stages

No.	GN	Protein name	Cluster	AD:NC	CIS:NC	ICC:NC
1	DMBT1	Deleted in malignant brain tumors 1 protein	5	3.12	3.43	4.82
2	S100A9	Protein S100-A9	5	2.56	2.52	4.67
3	CLC	Galectin-10	5	4.21	2.50	4.54
4	S100A8	Protein S100-A8	5	1.51	1.46	3.72
5	MPO	Myeloperoxidase	6	1.22	0.09	3.12
6	OLFM4	Olfactomedin-4	5	3.89	3.01	3.02
7	LDHA	L-lactate dehydrogenase A chain	5	1.93	3.69	2.92
8	SERPINB5	Serpin B5	5	4.32	2.69	2.78
9	EPX	Eosinophil peroxidase	5	3.16	2.82	2.73
10	COL12A1	Collagen alpha-1(XII) chain	5	0.57	4.35	2.68
11	TNC	Tenascin	5	-0.48	4.41	2.33
12	HNRNPA1	Heterogeneous nuclear ribonucleoprotein A1	5	2.69	3.55	2.16
13	MUC2	Mucin-2	2	-0.80	-5.21	-0.39
14	CA2	Carbonic anhydrase 2	2	-2.86	-5.08	-0.62
15	DCN	Decorin	4	-3.67	-2.03	-1.06
16	COL14A1	Collagen alpha-1(XIV) chain	2	-4.25	-3.42	-1.06
17	LUM	Lumican	4	-3.90	-1.40	-1.21
18	CA1	Carbonic anhydrase 1	2	-2.63	-4.96	-1.74
19	ITLN1	Intelectin-1	2	-2.18	-5.00	-1.91
20	ASPN	Asporin	4	-3.59	-1.36	-1.91
21	OGN	Mimecan	2	-4.35	-4.71	-2.29
22	GSTP1	Glutathione S-transferase P	7	-1.36	-1.06	-2.73
23	CKB	Creatine kinase B-type	2	-1.26	-2.98	-2.76
24	PFN1	Profilin-1	7	-0.47	0.30	-2.84
25	CHGA	Chromogranin-A	2	-3.44	-4.84	-3.20
26	TPI1	Triosephosphate isomerase	7	-0.88	-2.32	-3.75

the proteins in clusters 2 and 7 were associated with "O_2/CO_2 exchange in erythrocytes", "glutathione synthesis and recycling," and "TCA cycle" (Figure 4, Supplementary Tables 4 and 5). Interestingly, the abundance of proteins in clusters 8 and 9, which were mainly involved in pathways related with integrin and complement, were reduced first, and then increased. These proteins may exert different or even opposite functions at different stages of colorectal carcinogenesis through the associated pathways. The DEPs in each cluster were involved in multiple pathways, which indicated that multiple cellular pathways participated in the carcinogenic process, implying the complexity of the process.

Immunohistochemistry

Four of the top ranked proteins (DMBT1, S100A9, Galectin-10, and S100A8), which had expression levels that were progressively up-regulated during colorectal carcinogenic process, were chosen for immunohistochemical verification (Table 2). An independent set of archival tissue specimens including NC, AD, CIS and ICC were used for detection of the expression levels of the four proteins by immunohistochemistry. As shown in Figure 5 and Supplementary Table 3, expression levels of the four proteins significantly increased from early stage, AD, until late stage, ICC, but their expression patterns were not identical. For example, although Galectin-10 expression increased in all pathological stages ($P < 0.05$ for AD *vs* NC, CIS *vs* NC, and ICC *vs* NC), the expression level was lower at CIS than at AD or ($P < 0.05$ for CIS *vs* AD and ICC *vs* CIS). As another example, S100A8 expression was significantly higher at ICC than at AD or CIS ($P < 0.05$ for ICC *vs* AD and ICC *vs* CIS), although it also increased in all three pathological stages ($P < 0.05$ for AD *vs* NC, CIS *vs* NC and ICC *vs* AD).

DISCUSSION

Colorectal carcinogenesis has been a typical model for multistage carcinogenesis. The colorectal carcinogenic process includes several typical pathological stages: AD, CIS and ICC. Previous studies mostly focused on genes and acquired a great deal of information supporting the multistage model of colorectal carcinogenesis. With the advent of proteomics, researchers have made efforts to study alterations of the proteomes between certain stages of carcinogenesis[30,31]. Compared to previous reports on colorectal carcinogenesis, our current study mainly investigated the characteristics and dynamics of DEPs throughout multiple typical stages of the colorectal carcinogenic processes. Protein quantification by iTRAQ is a very useful technique to monitor relative changes in proteins in a variety of settings, such as multiple stages of cancer development. Some limitations of iTRAQ technique include underestimation of ratios, limited dynamic range (fold changes of < 2 orders of magnitude), and relatively expensive reagents[32,33]. Since iTRAQ underestimates ratios, we expect that the actual ratio change of up-regulation or down-regulation would be more than that we reported.

There were 199 DEPs founded between AD *vs* NC. GO enrichment analysis indicated that proteins

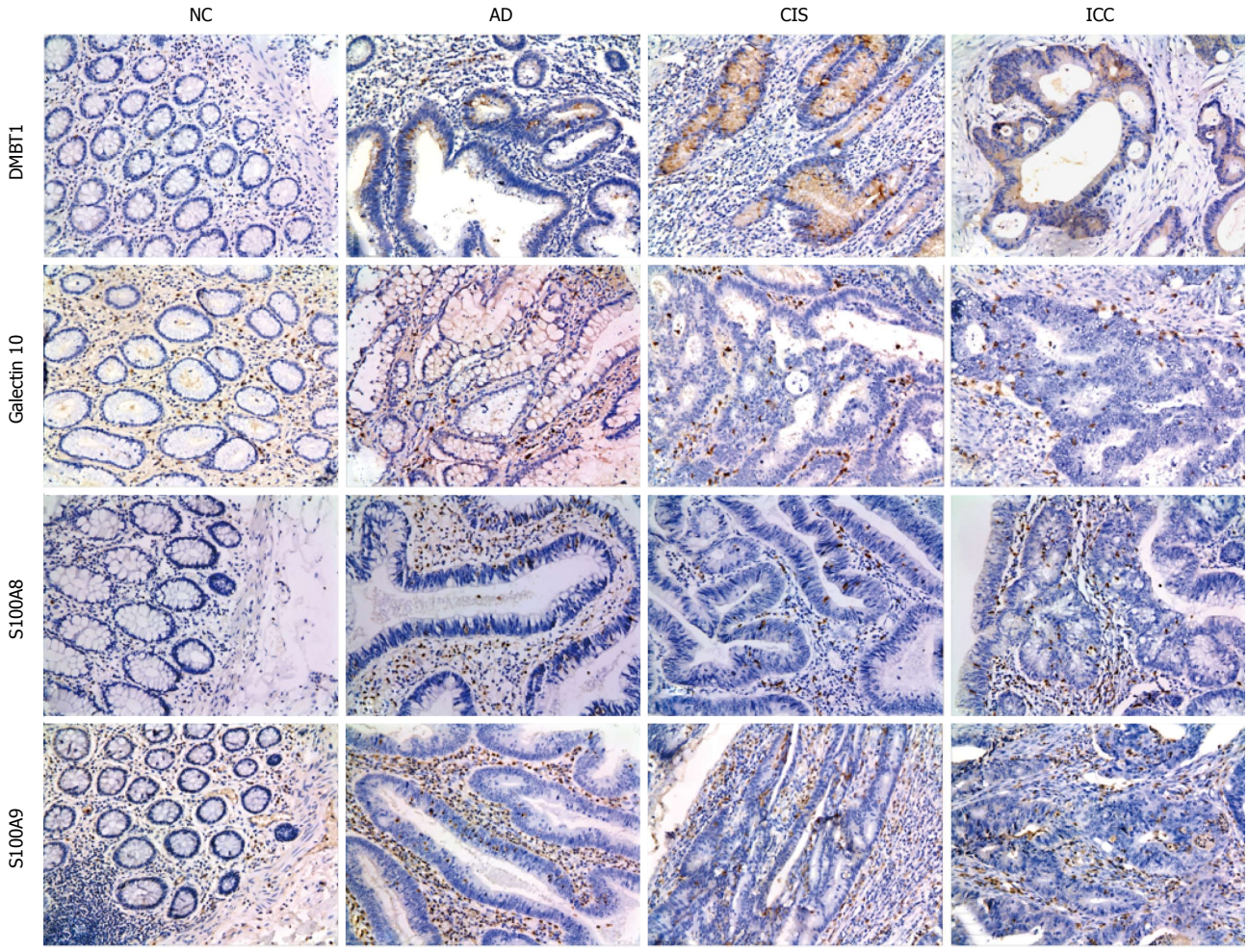


Figure 5 Representative results of immunohistochemistry show the expression of DMBT1, S100A9, Galectin-10 and S100A8 in the NC, AD, CIS and ICC (Original magnification, × 200). DMBT1 immunostaining in NC, AD, CIS and ICC. Negative staining was observed in NC, moderate in AD tissues, and strong cytoplasmic staining in CIS and ICC tissues. S100A9 immunostaining in NC, AD, CIS and ICC. Negative staining was found in NC, weak intralesional staining in AD, moderate intralesional staining in CIS and strong intralesional staining in ICC. Galectin-10 immunostaining in NC, AD, CIS and ICC. Negative staining was found in NC, weak staining in AD and CIS, and moderate staining in ICC. S100A8 immunostaining in NC, AD, CIS and ICC. Negative staining was observed in NC, moderate intralesional staining in AD and CIS, and strong intralesional staining in ICC.

associated with DNA replication, translation, protein complex biogenesis and assembly were significantly up-regulated in AD, whereas proteins associated with blood coagulation, response to external stimulus, complement activation and cell adhesion, *etc.*, were down-regulated in AD. Previous genetic analysis by Suppression Subtractive Hybridization (SSH) also found that genes involved in DNA replication, complement activation and cell adhesion were significantly differentially expressed in AD[34]. This is in accordance with our findings at the protein level.

Previous studies compared the proteomes between colorectal adenomas and carcinomas, and found that the DEPs participated in RNA processing, translation, and cell adhesion[35,36]. In the present study, we also showed that during the transition from AD to CIS, the DEPs were associated with cell-cell adhesion, cell-matrix adhesion, translation and RNA metabolic processing. In addition, our results showed that the up-regulated proteins were associated with cell cycle and cellular component movement, while the down-regulated proteins were associated with respiratory electron transport chain and fatty acid beta-oxidation. Previous studies using genome-wide mRNA expression profile analysis showed that the pathway of fatty acid metabolism is down-regulated in CRC compared to adenomas[37]. In addition to CRC, proteins involved in fatty acid β-oxidation were also found to be down-regulated in pancreatic cancer cells[38,39].

Between CIS and ICC, the proteins associated with complement activation were up-regulated in ICC. The multiple roles that complement proteins play in carcinogenesis, including functions that facilitate cancer metastasis such as promotion of angiogenesis, invasion and migration, have been reviewed[40,41]. Furthermore, recent studies demonstrate that activation of the complement C5 component C5a and its receptor C5aR can promote cancer cell invasion[42,43].

DMBT1 (deleted in malignant brain tumours 1) gene is located in 10q25.3-q26.1, a region with frequent LOH

in many types of human cancers. Therefore, DMBT1 was proposed to be a tumour suppressor[44]. DMBT1 deletion occurs in brain tumours[45], and down-regulation of DMBT1 was reported in mammary tumours, oral squamous cell carcinoma, skin cancers and other tumour types[46,47]. However, there are also a number of reports showing that DMBT1 was up-regulated in cancers. For example, Dolznig *et al*[48] found that DMBT1 expression was increased in colonic samples compared to normal controls. In our present study, we also found that DMBT1 expression was up-regulated from early to late stages of colonic carcinogenesis. In addition to CRC, DMBT1 expression was also up-regulated during oesophageal carcinogenesis[49]. Since DMBT1 expression was up-regulated in some cancer types while down-regulated in others, the mechanism by which DMBT1 contributes to carcinogenesis could be tissue-specific, and needs more investigation.

S100A9 and S100A8 proteins are members of a family of Ca2+ binding proteins. Both proteins are often co-expressed and form a heterodimer to exert their biological functions. Overexpression of S100A8 and S100A9 has been associated with carcinogenesis[50-52]. For example, Stulík *et al*[53] reported that S100A9/A8 were up-regulated in human colon carcinoma. S100A9/A8 could activate MAPK and NF-κB pathways in colon tumour progression[50]. Our results showed that S100A9/A8 expression was gradually up-regulated during carcinogenesis. The tendency of both protein expression levels during colonic carcinogenesis was similar, which was consistent with the performance of their biological function as a heterodimer. Our study suggested that both proteins are associated with colorectal carcinoma progression, which supported the therapeutic strategies of blocking S100A9/A8 activity for either inflammatory diseases or cancer[52].

Galectins are a family of proteins characterized by their binding specificity for β-galactoside sugars. The best understood galectin in cancer is Galectin-3. Galectin-3 has been shown to play important roles in tumorigenesis processes, including transformation, metastasis and invasion[54,55]. As for Galectin-10, Ågesen *et al*[56] demonstrated that Galectin-10 was the most differentially expressed gene, with 10-fold higher expression in early- *vs* late-onset CRC, and was important in the development of early-onset CRC.

The present work investigated for the first time the dynamic expression patterns of DEPs in multistage carcinogenesis of CRC using quantitative proteomic methods. We systematically compared the characteristics and dynamics of the expressed proteins across the various stages of colorectal carcinogenesis. The findings reported here provide a basis for discovery of candidate biomarkers for early diagnosis of CRC, and give clues for further investigation of the mechanisms of colorectal carcinogenesis and for discovery of new therapeutic targets.

COMMENTS

Background

Colorectal cancer is the third leading cause of cancer death in the world. Colorectal carcinogenesis is a multistep and complicated process, from normal colonic mucosa, adenoma, carcinoma *in situ*, and ultimately to invasive colorectal carcinoma.

Research frontiers

In recent years, much progress has been made in understanding genetic changes in the colonic carcinogenesis process, and many studies have been conducted to analyze differentially expressed proteins between certain stages of colorectal carcinogenesis

Innovations and breakthroughs

In this study, proteomic analysis was used to identify differentially expressed proteins in the human colonic epithelial carcinogenic process, including normal colon, adenoma, carcinoma *in situ* and invasive carcinomas tissues. A total of 326 proteins were identified to be differentially expressed. The differential expression of four proteins (DMBT1, S100A9, Galectin-10, and S100A8) was validated using immunohistochemistry.

Applications

These findings give insights into our understanding of the mechanisms of colorectal carcinogenesis. In addition, these studies provide a list of differentially expressed protein as potential biomarkers.

Peer-review

This paper provides practical and novel proteomic information that is currently not known with respect to the expression of various proteins during colon carcinogenesis. It is well written, and data presented are interesting.

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ORIGINAL ARTICLE

Basic Study

Elevated serum interleukin-38 level at baseline predicts virological response in telbivudine-treated patients with chronic hepatitis B

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Abstract

AIM: To investigate serum interleukin (IL)-38 level and its clinical role in predicting virological response (VR) to telbivudine (LdT) in patients with chronic hepatitis B (CHB).

METHODS: The study participants were divided into two groups; one group consisted of 43 healthy controls (HCs) and the other group consisted of 46 patients with hepatitis B e antigen-positive CHB. All patients were administered 600 mg of oral LdT daily for 52 wk, and they visited physicians every 12 wk for physical examination and laboratory tests. Serum IL-38 levels were determined using ELISA. The concentrations of serum Th1- and Th2-type cytokines were measured using the cytometric bead array (CBA) method.

RESULTS: Serum levels of IL-38 at baseline in all patients were higher than those in HCs [306.97 (123.26-492.79) pg/mL *vs* 184.50 (135.56-292.16) pg/mL, $P = 0.019$]; the levels returned to normal after the first 12 wk of treatment with LdT [175.51

(103.90-331.91) pg/mL *vs* 184.50 (135.56-292.16) pg/mL, $P > 0.05$]. Serum IL-38 levels at baseline were positively associated with serum aspartate aminotransferase levels in patients with CHB ($r = 0.311$, $P = 0.036$). Higher levels of serum IL-38 at baseline were associated with a greater probability of VR to LdT treatment at 24 wk (48.15% *vs* 15.79%, $P = 0.023$) and 52 wk (66.67% *vs* 36.84%, $P = 0.044$). The levels of serum IL-38 in patients with primary non-response at week 12 after treatment initiation were lower than those in patients with primary response [64.44 (49.85-172.08) pg/mL *vs* 190.54 (121.35-355.28) pg/mL, $P = 0.036$]. Serum IL-38 levels were correlated with serum IL-6 and IL-12 levels in patients with CHB during treatment with LdT.

CONCLUSION: Elevated serum IL-38 levels in untreated CHB patients reflect ongoing liver injury. Higher serum IL-38 levels before treatment indicate a greater probability of VR to LdT treatment.

Key words: Alanine aminotransferase; Aspartate aminotransferase; Interleukin-6; Interleukin-12; Interleukin-38; Chronic hepatitis B; Primary non-response; Virological response

Core tip: This is the first study detailing kinetic changes in serum interleukin-38 (IL-38) levels during chronic hepatitis B (CHB). Higher pretreatment serum IL-38 levels are associated with a greater probability of virological response to telbivudine treatment. Elevated levels of serum IL-38 in untreated patients with CHB reflect ongoing liver injury, which is an indirect indicator of vigorous endogenous clearance of hepatitis B virus infection. Our findings suggest that clear signs of viral clearance at baseline may predict a favorable response to treatment of CHB using nucleoside analogs.

Wang HJ, Jiang YF, Wang XR, Zhang ML, Gao PJ. Elevated serum interleukin-38 level at baseline predicts virological response in telbivudine-treated patients with chronic hepatitis B. *World J Gastroenterol* 2016; 22(18): 4529-4537 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i18/4529.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i18.4529

INTRODUCTION

Hepatitis B virus (HBV) infection is a serious global health concern, and approximately 240 million people have been chronically infected with HBV[1]. Patients with chronic hepatitis B (CHB) have a high risk of developing liver cirrhosis and hepatocellular carcinoma[2].

Previous studies have revealed that HBV infection may suppress the immune system, and that Th1 and Th2 cells and their respective cytokines are possibly involved in the pathogenesis of CHB[3,4]. Telbivudine (LdT), a nucleoside analog (NA), not only inhibits viral replication, but also increases cytokine production by the Th1 cell subpopulation, which could contribute to its antiviral efficacy[5]. Th2 cells secrete interleukin (IL)-10 and IL-6, which are involved in liver inflammation in CHB[6,7]. Members of the IL-1 cytokine family are also associated with acute and chronic inflammation, and they facilitate the differentiation and function of polarized innate and adaptive lymphoid cells[8,9]; they likely participate in CHB infection. For example, it has been reported that IL-37, an anti-inflammatory cytokine, was a part of the immune response in patients with CHB who showed hepatitis B e antigen (HBeAg) seroconversion, and that both serum IL-33 and IL-37 levels are associated with liver injury in these patients[10,11].

IL-38 (IL-1F10) is a newly characterized cytokine of the IL-1 family, and it is expressed in the basal epithelia of the skin and in the proliferating tonsillar B cells[12]. It was suggested that IL-38 may have anti-inflammatory properties, since it shares some sequence homology with IL-1Ra (41%) and IL-36Ra (43%)[8]. This assumption was first supported by a study that showed that a recombinant IL-38 bound to the IL-36 receptor and inhibited the expression of IL-17, IL-22, and IL-8 in human peripheral blood mononuclear cells (PBMCs) under inflammatory conditions. It was further suggested that IL-38 might function as a partial IL-36 receptor antagonist[13]. Another study observed a marked increase in lupus-associated pro-inflammatory mediators following silencing of endogenous IL-38 in PBMCs, which supported the assumed anti-inflammatory function of IL-38[14]. Furthermore, IL-38 was upregulated in patients with primary Sjogren's syndrome, and it was suggested to counteract the imbalanced activation of IL-36[15]. Genetic association studies indicated that IL-38 polymorphisms are linked with a high frequency of psoriatic arthritis, ankylosing spondylitis, and rheumatoid arthritis in the population, suggesting that IL-38 is a likely component in the pathogenesis of these inflammatory diseases[8,16,17].

NAs and interferons (IFNs) are the currently available antiviral agents for treating CHB[18]. LdT is a synthetic thymidine NA that shows potent inhibition of HBV replication[19]. Recent studies have shown that treatment with LdT not only suppresses HBV replication, but also modulates the immune response in treated CHB patients[20].

To our knowledge, there are no available data regarding the expression or function of IL-38 in CHB. In this study, we quantified the kinetic changes in serum IL-38 level and investigated the potential correlation between the levels of HBV-related biochemical markers, Th1/Th2 type cytokines, and serum IL-38 levels during LdT treatment of patients with CHB. In addition, we discussed the implications of our findings.

MATERIALS AND METHODS

Patients

A total of 46 patients with CHB were recruited at the First Hospital of Jilin University from September 2012 to October 2014. Patients with CHB showed positive results for hepatitis B surface antigen (HBsAg), HBeAg, and HBV DNA for at least 12 mo. Another 43 gender-, age- and ethnicity-matched healthy controls (HCs) were recruited from the Physical Examination Center of our hospital during the same period. Individuals who showed seropositivity of hepatitis C, G, or D virus, those infected by HIV-1 or those with autoimmune liver disease were excluded from the study. None of the participants had received immunosuppressive or antiviral therapy during the 12 mo preceding their inclusion in the study, and none of them reported a history of exposure to known hepatotoxins. The demographic and clinical characteristics of the patients are summarized in Table 1. Written informed consent was obtained from each study participant; the experimental protocol was established according to the guidelines of the 1975 Declaration of Helsinki and was approved by the Human Ethics Committee of Jilin University, China.

Patients were administered 600 mg LdT (Novartis Pharmaceuticals, Beijing, China) daily for 52 wk, and they visited the outpatient department of the hospital every 12 wk for physical examination and laboratory tests. All patients were followed up for one year. Peripheral blood samples were obtained and serum samples were separated; the samples were stored at -80 ℃ until use.

Measurement of serum IL-38 by enzyme-linked immunosorbent assay

Serum concentrations of IL-38 in patients and HCs were determined by enzyme-linked immunosorbent assay [human IL-38 enzyme-linked immunosorbent assay (ELISA) kit; CUSABIO Life Sciences, Wuhan, Hunan province, China]. Briefly, individual sera were subjected to ELISA, and the concentrations of serum IL-38 in individual samples were calculated using the standard curve established with the recombinant IL-38 provided. The detection limit of IL-38 ranged between 31.25 and 2000 pg/mL.

Cytometric bead array of serum Th1- and Th2-type cytokines

The concentrations of serum Th1- and Th2-type cytokines (IFN-γ, TNF-α, IL-2, IL-4, IL-12, IL-10, and IL-6) were determined by cytometric bead array (CBA) according to the manufacturer's protocol (BD Biosciences, San Jose, CA, United States), with minor modifications. Briefly, 25 μL of individual serum was used in duplicate for analysis. The concentrations of serum cytokines were quantified using the CellQuest Pro and CBA software (Becton Dickinson, San Jose, CA, United States) on an FACSAria Ⅱ flow cytometer (BD Biosciences).

Table 1 Demographic and clinical characteristics of study subjects at baseline

Parameters	Patients with CHB (n = 46)	Healthy controls (n = 43)
Age (yr)	34.5 (23.75-38.25)	34 (22-40)
Sex (M/F)	38/8	34/9
HBV DNA load (log_{10} IU/mL)	7.71 (6.98-8.10)	NA
ALT level (U/L)	117.35[a] (74.50-241.48)	14 (6-22)
AST level (U/L)	96.75[a] (57.25-176.25)	12 (10-30)
HBsAg level (IU/mL)	7821.43 (3337.23-20737.14)	NA
HBsAg, pos/neg	46/0	0/43
HBeAg, pos/neg	46/0	0/43

Data are represented as median and interquartile range (IQR) or real case number. Normal values: ALT ≤ 50 IU/L; AST ≤ 40 IU/L; HBV DNA ≤ 1.78 log_{10} IU/mL (60 IU/mL). [a]$P < 0.05$ *vs* the HCs. The undetectable HBV DNA loads were recorded as those ≤ 60 IU/mL. CHB: Chronic hepatitis B; HBV: Hepatitis B virus; NA: Not applicable; ALT: Alanine transferase; AST: Aspartate transferase; HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B e antigen; pos: Positive; neg: Negative.

Serological analysis of HBV markers

Serum HBsAg, hepatitis B surface antibody (HBsAb), HBeAg, and hepatitis B e antibody (HBeAb) were detected by chemiluminescent microparticle immunoassay (CMIA), using an Abbott i2000 automated chemiluminescent immunoassay analyzer (Abbott Laboratories, Abbott Park, IL, United States). The levels of serum alanine transferase (ALT) and aspartate transferase (AST) were detected using a Biochemistry Automated Analyzer (Roche Diagnostics, Branchburg, New Jersey, United States). Serum HBV DNA was measured by quantitative PCR using a luciferase-based detection kit, following the manufacturer's protocols (Roche). The detection limit of viral DNA was 60 IU/mL (HBV DNA ≤ 60 IU/mL was considered undetectable).

Statistical analysis

Quantitative data were expressed as median and interquartile range (IQR). Differences between independent groups were analyzed by Mann-Whitney *U* test or Student's *t*-test when appropriate and differences between related groups were analyzed by Wilcoxon signed ranks test. The correlation between variables was evaluated using Spearman's rank correlation test. Receiver operating characteristic curves were constructed to identify optimal cutoff values for predicting virological response (VR) to treatment. Fisher's exact tests were carried out to compare the rates of VR, HBeAg loss, ALT normalization and HBsAg loss. Binary logistic regression was used to determine predictors of VR. All statistical analyses were performed by the SPSS software (version 18.0). *P*-value < 0.05 was considered as statistically significant.

Table 2 Dynamics of clinical parameters in patients with chronic hepatitis B during LdT treatment

Parameters	Baseline	3 mo	6 mo	9 mo	13 mo
HBV DNA load (log_{10} IU/mL)	8.41 (7.68-8.80)	3.79[a] (3.20-5.14)	3.04[a] (2.48-3.98)	2.81[a] (2.48-3.10)	2.48[a] (2.48-3.50)
ALT level (U/L)	117.35 (74.50-241.48)	36.00[a] (23.93-85.50)	27.00[a] (22.00-38.00)	27.50[a] (23.00-34.25)	29.50[a] (24.00-55.00)
AST level (U/L)	96.75 (57.25-176.25)	34.00[a] (28.00-55.25)	26.00[a] (22.00-31.25)	24.50[a] (19.75-30.00)	27.00[a] (22.75-43.25)
HBsAg level (IU/mL)	7821.43 (3337.23-20737.14)	4214.99[a] (2034.48-10170.46)	5153.19[a] (2039.45-10671.30)	4414.88[a] (2317.96-10084.21)	4210.48[a] (1664.73-9823.78)

Data are represented as median and interquartile range (IQR). Normal values: ALT ≤ 50 IU/L; AST ≤ 40 IU/L; HBV DNA ≤ 1.78 log_{10} IU/mL (60 IU/mL). [a]$P < 0.05$ *vs* baseline value. The undetectable HBV DNA loads were recorded as those ≤ 60 IU/mL. CHB: Chronic hepatitis B; HBV: Hepatitis B virus; ALT: Alanine transferase; AST: Aspartate transferase; HBsAg: Hepatitis B surface antigen.

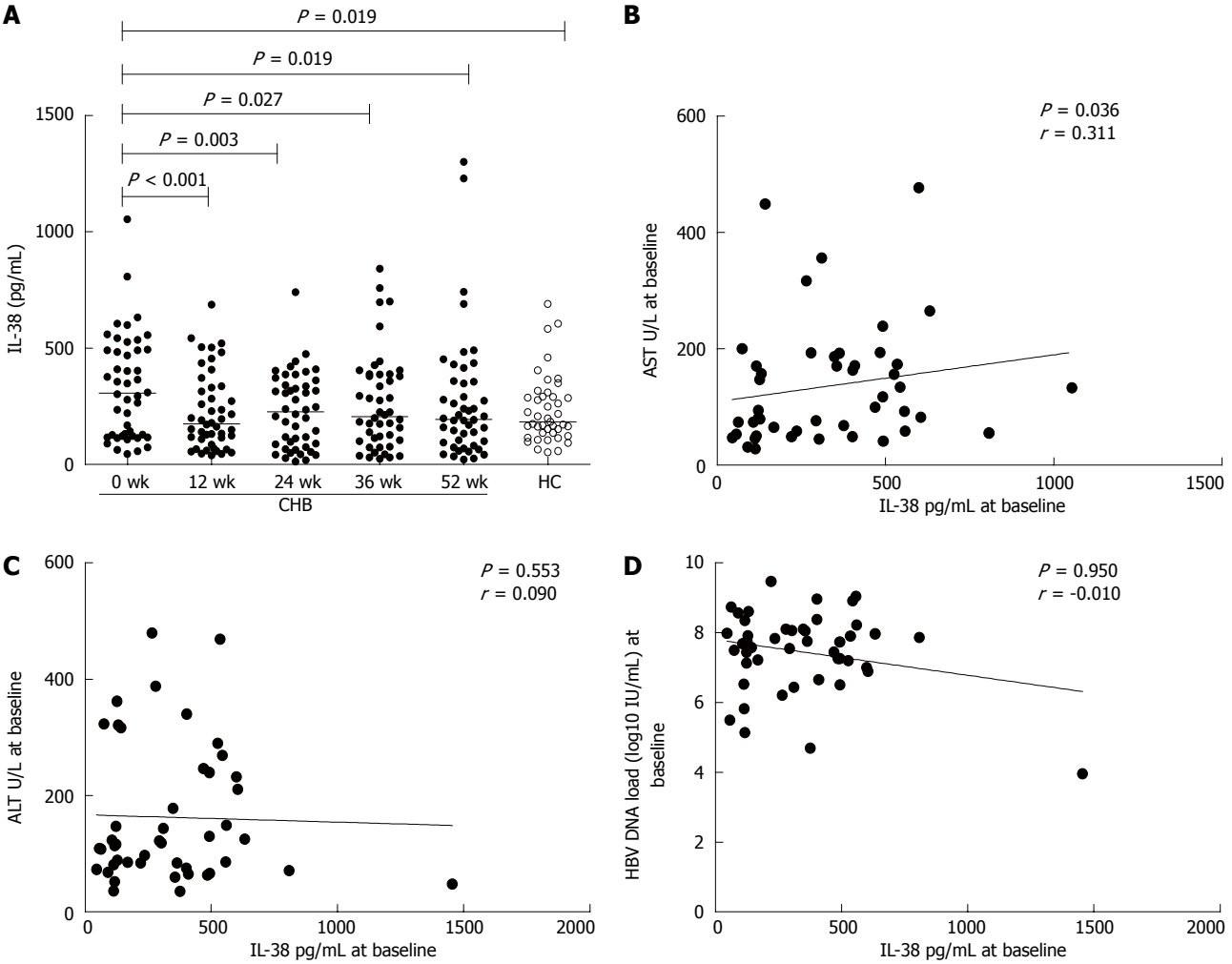

Figure 1 Kinetic changes in serum interleukin-38 levels of patients with chronic hepatitis B during treatment with telbivudine and association of serum interleukin-38 with serum aspartate transferase, alanine transferase, hepatitis B virus DNA loads prior to treatment. A: Kinetic changes in the levels of serum IL-38 in HCs and patients with CHB at baseline and at 12, 24, 36, and 52 wk of LdT treatment. IL-38 levels were higher in patients with CHB compared with HCs at baseline [306.97 (123.26-492.79) pg/mL *vs* 184.50 (135.56-292.16) pg/mL; $P = 0.019$]. They were reduced at week 12, 24, 36, and 52 of LdT treatment ($P < 0.001$, $P = 0.003$, $P = 0.027$, $P = 0.019$), and the levels of serum IL-38 in patients with CHB were no longer different from that of the HCs after 12 wk of LdT treatment. B: Serum AST levels correlated with serum IL-38 levels at baseline. C and D: Serum IL-38 levels did not correlate with serum ALT levels or with serum HBV DNA loads at baseline. IL-38: Interleukin-38; HCs: Healthy controls; CHB: Chronic hepatitis B; LdT: Telbivudine; ALT: Alanine transferase; AST: Aspartate transferase; HBV: Hepatitis B virus.

RESULTS

HBV-related biochemical markers in patients with CHB before and during treatment with LdT

There was no significant difference in the distribution of age or gender between patients with CHB and HCs (Table 1).

HBV DNA and HBsAg levels gradually decreased in response to the LdT treatment and were lower than the levels before treatment. Both ALT and AST

Table 3 Comparison of demographic and clinical characteristics of patients with high and low interleukin-38 levels before treatment

Parameter	High IL-38 level before treatment (n = 27)	Low IL-38 level before treatment (n = 19)
Age (yr)	35 (23-38)	34 (25-40)
Sex (M/F)	22/5	16/3
HBV DNA load (log_{10} IU/mL)	7.74 (6.89-8.10)	7.69 (7.14-8.36)
ALT level (U/L)	130.00 (71.00-246.50)	108.00 (81.00-147.00)
AST level (U/L)	156.00[c] (76.00-193.00)	74.00 (49.00-147.00)
HBsAg level (IU/mL)	6641.55 (4437.10-18631.33)	10222.92 (2657.06-24877.19)

Data are represented as median and interquartile range (IQR) or real case number. Normal values: ALT ⩽ 50 IU/L; AST ⩽ 40 IU/L; HBV DNA ⩽ 1.78 log_{10} IU/mL (60 IU/mL). [c]P < 0.05 *vs* the group of patients with low serum IL-38 levels before LdT treatment. The undetectable HBV DNA loads were recorded as those ⩽ 60 IU/mL. High IL-38 level: Serum IL-38 ⩾ 250 pg/mL; Low IL-38 level: Serum IL-38 < 250 pg/mL; IL-38: Interleukin-38; HBV: Hepatitis B virus; ALT: Alanine transferase; AST: Aspartate transferase; HBsAg: Hepatitis B surface antigen; M: Male; F: Female.

levels also decreased during treatment (Table 2). VR was defined as undetectable HBV DNA (⩽ 60 IU/mL). Primary non-response (PNR) was defined as a decrease in HBV DNA of less than 1 log10 IU/mL from baseline at 12 wk after treatment initiation[21]. Of the 46 patients, 5 showed PNR and the remaining 41 patients showed primary response (PR) at week 12. At weeks 24 and 52 of LdT therapy, 16 (35%) and 25 (54%) patients with CHB showed VR, respectively.

Kinetic changes in serum IL-38 levels of patients with CHB during treatment with LdT and association of serum IL-38 with serum AST prior to treatment

The kinetic changes in serum IL-38 levels during treatment were determined. Serum IL-38 levels at the baseline and at 12, 24, 36, and 52 wk were 306.97 (123.26-492.79) pg/mL, 175.51 (103.90-331.91) pg/mL, 226.53 (84.33-346.47) pg/mL, 205.91 (103.48-387.16) pg/mL, and 194.79 (90.71-356.48) pg/mL, respectively. As shown in Figure 1, IL-38 levels were higher in patients with CHB compared with HCs at baseline [306.97 (123.26-492.79) pg/mL *vs* 184.50 (135.56-292.16) pg/mL, P = 0.019, Figure 1A], and they were reduced at week 12, 24, 36, and 52 of LdT treatment (P < 0.001, P = 0.003, P = 0.027, P = 0.019, Figure 1A). In addition, the levels of serum IL-38 in patients with CHB were no longer different from that of the HCs after 12 wk of LdT treatment, since a quick reduction was observed only during the first 12 wk.

Correlation analysis of serum IL-38 with ALT and AST levels showed that serum IL-38 levels at baseline were positively associated with serum AST levels (P = 0.036, r = 0.311, Figure 1B) but not with serum ALT levels (P > 0.05, Figure 1C) in patients with CHB. No significant correlation was found between serum IL-38 and serum AST or ALT values during LdT therapy (data not shown).

In addition, there was no significant association between serum IL-38 and HBV DNA levels prior to LdT treatment (P > 0.05, Figure 1D), and no significant correlation during LdT therapy (data not shown).

Pretreatment serum IL-38 levels in patients with CHB were associated with antiviral therapy outcomes

We generated receiver operating characteristic curves to decide optimal cut-off values of serum IL-38 at baseline to predict VR. Using the serum IL-38 levels at baseline, we divided the 46 patients with CHB into two groups: high level group (HG; serum IL-38 ⩾ 250 pg/mL, n = 27) and low level group (LG; serum IL-38 < 250 pg/mL, n = 19). The pretreatment characteristics of the two groups are shown in Table 3. There was no significant difference in the distribution of age or gender, and serum HBV DNA, ALT, and HBsAg levels between HG and LG. However, the serum AST levels differed between the groups. Interestingly, the serum HBV DNA loads in HG were lower than those in LG at week 12, 24, 36, and 52 post initial LdT treatment (P = 0.041, 0.003, 0.003, 0.020, Figure 2A), suggesting a better response in patients with high serum levels of IL-38. No significant difference in serum HBsAg levels was noted between the groups during LdT therapy (P > 0.05 at week 12, 24, 36, and 52, Figure 2B).

The VR rates were compared between HG and LG, and it was observed that at 24 wk, VR was achieved in 13 patients (48.15%) in HG and 3 patients (15.79%) in LG. Higher serum IL-38 levels at baseline were associated with greater VR (OR = 4.95, 95%CI: 1.17-21.03, P = 0.023). At 52 wk, 18 patients (66.67%) in HG and 7 patients (36.84%) in LG achieved VR. The IL-38 levels at baseline elicited different responses to therapy (OR = 3.43, 95%CI: 1.00-11.71, P = 0.044). Treatment outcomes, including HBeAg elimination, ALT normalization and HBsAg loss, between patients with high and low baseline IL-38 levels were also compared. As shown in Table 4, there were no significant differences in HBeAg elimination, ALT normalization and HBsAg loss between HG and LG. According to our data, an elevated serum IL-38 level at baseline could predict better virological response.

The serum AST, ALT, HBsAg, and HBV DNA levels at baseline were analyzed by binary logistic regression and they were not associated with VR at week 24 or 52 of LdT treatment (data not shown).

Primary response was associated with high serum IL-38 levels

The levels of serum IL-38 in patients with CHB who showed PR were higher than those of CHB patients who showed PNR at 12 wk after LdT treatment [190.54 (121.35-355.28) pg/mL *vs* 64.44 (49.85-172.08) pg/mL, P = 0.036, Figure 3], while they did not differ at baseline.

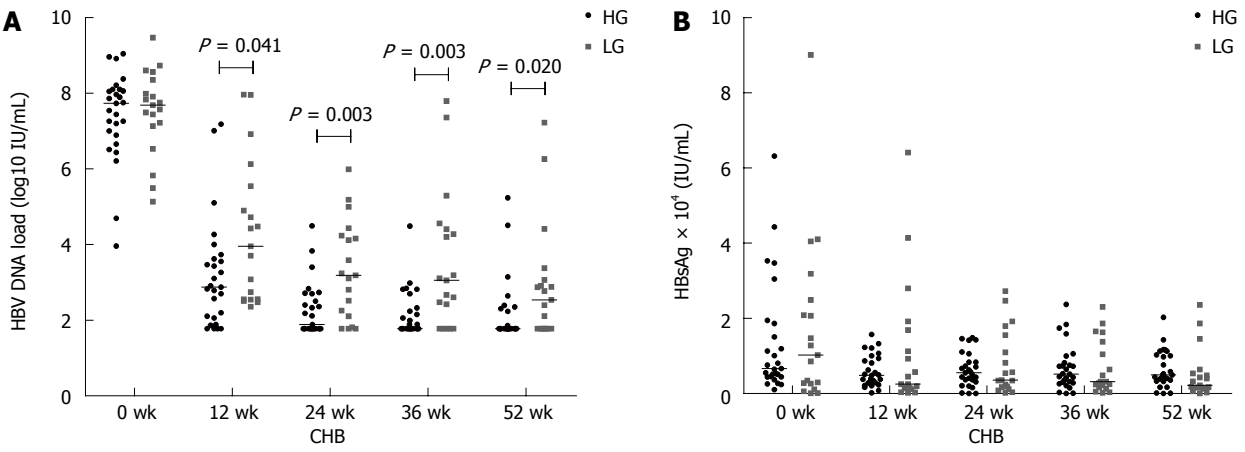

Figure 2 Comparison of serum hepatitis B virus DNA loads (A) and hepatitis B surface antigen levels (B) between high level group and low level group at baseline and at 12, 24, 36, and 52 wk of LdT treatment. A: The serum HBV DNA loads in HG were lower than those in LG at week 12, 24, 36, and 52 post initial LdT treatment (P = 0.041, 0.003, 0.003, 0.020). B: No significant difference in serum HBsAg levels was noted between the groups during LdT therapy. LdT: Telbivudine; HBV: Hepatitis B virus; HG: High level group; LG: Low level group; HBsAg: Hepatitis B surface antigen.

Table 4 Comparison of treatment outcomes between patients with high and low baseline interleukin-38 levels

Parameters	Week 24			Week 52		
	HG (n = 27)	LG (n = 19)	P value	HG (n = 27)	LG (n = 19)	P value
HBV DNA ≤ 60 IU/mL	13 (48.1)	3 (15.8)	0.023	18 (66.7)	7 (36.8)	0.044
HBeAg seroconversion	4 (14.8)	2 (10.5)	0.516	8 (29.6)	4 (21.1)	0.382
ALT normalization	25 (92.6)	15 (78.9)	0.182	21 (77.8)	12 (63.2)	0.225
HBsAg loss	0 (0.0)	0 (0.0)	NA	1 (3.7)	1 (5.3)	0.661

Normal values: ALT ≤ 50 IU/L; AST ≤ 40 IU/L; HBV DNA ≤ 1.78 log10 IU/mL (60 IU/mL). The undetectable HBV DNA loads were recorded as those ≤ 60 IU/mL. HG: High IL-38 level group, basement serum IL-38 ≥ 250 pg/mL; LG: Low IL-38 level, basement serum IL-38 < 250 pg/mL; IL-38: Interleukin-38; HBV: Hepatitis B virus; ALT: Alanine transferase; AST: Aspartate transferase; HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B e antigen; NA: Not applicable.

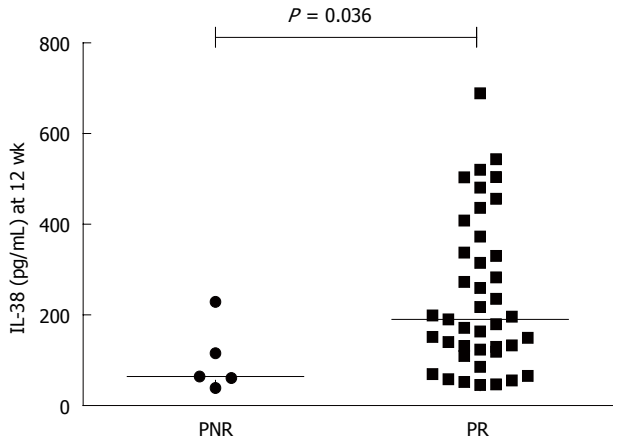

Figure 3 Comparison of serum interleukin-38 levels between patients with primary non-response and primary response at 12 wk post initial telbivudine treatment. The levels of serum IL-38 in patients with CHB who showed PR were higher than those of CHB patients showed PNR at 12 wk after LdT treatment [190.54 (121.35-355.28) pg/mL *vs* 64.44 (49.85-172.08) pg/mL, P = 0.036]. IL-38: Interleukin-38; CHB: Chronic hepatitis B; LdT: Telbivudine; ALT: Alanine transferase; AST: Aspartate transferase; HBV: Hepatitis B virus; PNR: Primary non-response; PR: Primary response.

Serum IL-38 levels correlated with IL-6 and IL-12 levels in patients with CHB during LdT treatment

As shown in Figure 4, the serum levels of IL-38 were positively associated with the levels of serum IL-6 at baseline, week 12, 24, 36, and 52 after starting LdT treatment (P = 0.003, r = 0.435; P = 0.002, r = 0.436; P = 0.001, r = 0.481; P < 0.001, r = 0.552; P = 0.008, r = 0.386; Figure 4A-E). The levels of serum IL-38 were positively associated with the levels of serum IL-12 at week 24, 36, and 52 of LdT treatment (P = 0.042, r = 0.301; P < 0.001, r = 0.515; P = 0.002, r = 0.451; Figure 4F-H).

DISCUSSION

To our knowledge, this is the first study detailing kinetic changes in serum IL-38 levels during CHB. We found that the levels of serum IL-38 at baseline in patients with CHB were higher than in HCs, which declined and returned to normal after 12 wk of LdT treatment. There was a positive correlation between IL-38 and AST levels at baseline. Patients with high serum levels of IL-38 showed a higher probability of VR than patients with low serum levels of IL-38. Further, the serum HBV DNA level in patients with high baseline IL-38 levels was reduced after 12 wk of LdT treatment as compared to patients with low baseline IL-38 levels.

Our findings indicated that IL-38 level may be a

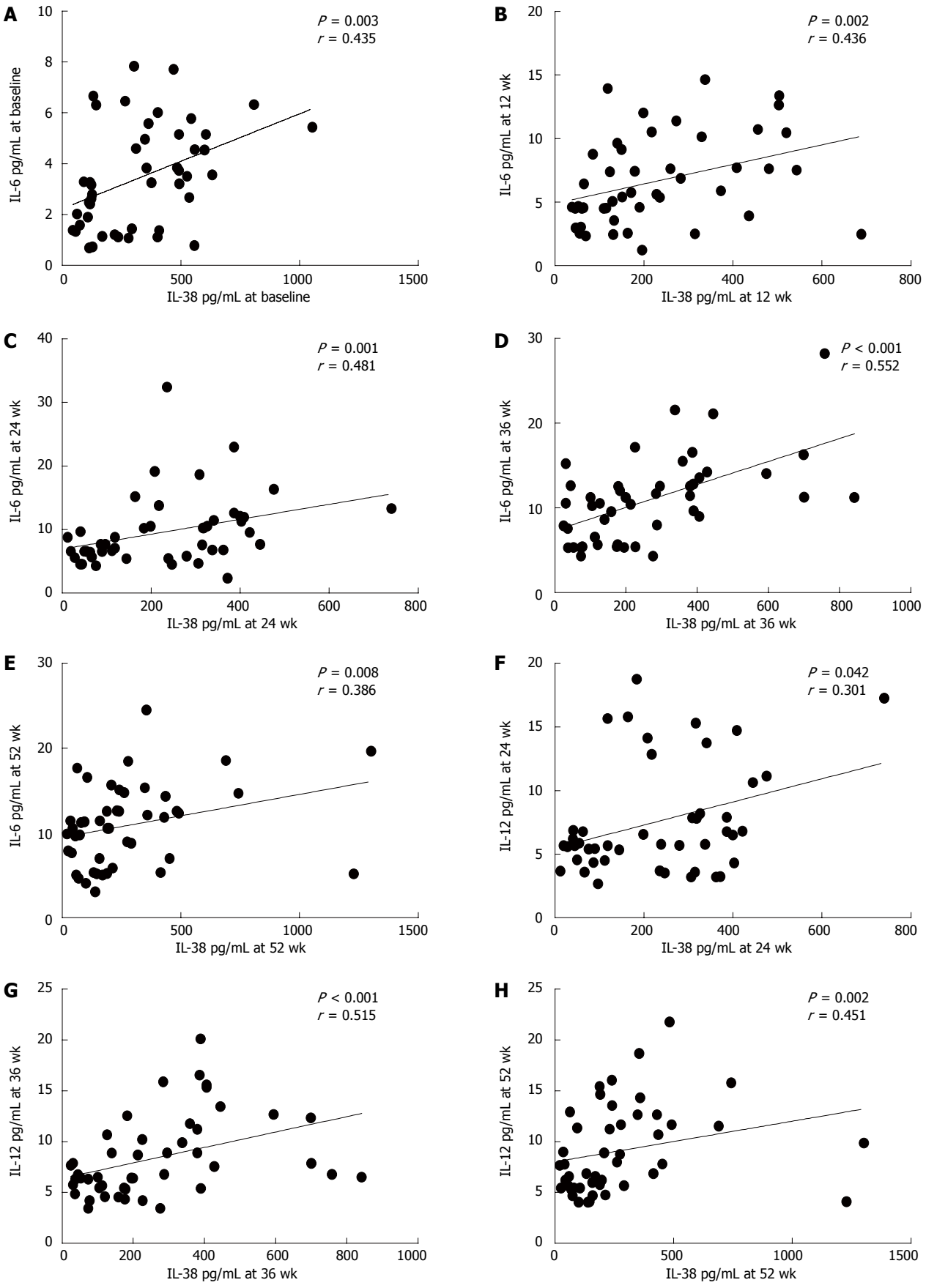

Figure 4 Correlation of serum interleukin-38, Th1- and Th2-type cytokines levels. A-E: Serum IL-38 levels correlated with serum IL-6 levels in patients with CHB during LdT treatment at baseline, and at 12, 24, 36, and 52 wk, respectively; F-H: Serum IL-38 levels correlated with serum IL-12 levels in patients with CHB during LdT treatment at 24, 36, and 52 wk, respectively. IL-38: Interleukin-38; CHB: Chronic hepatitis B; LdT: Telbivudine.

marker reflecting liver injury, since elevated IL-38 at baseline was in parallel with elevated AST, an indicator of liver injury[22]. However, we could not confirm whether elevated IL-38 level was a primary trigger for the liver injury or a response to it. Nevertheless, the elevated IL-38 level indicates hepatic necroinflammation to a certain extent. An active hepatic necroinflammation is a result of destruction of infected hepatocytes, and it can be viewed as viral clearance. Based on this information, we can explain why patients with high IL-38 and AST levels at baseline showed much better VR to LdT treatment, because these patients showed more vigorous endogenous clearance of HBV infection in addition to the antiviral effect of LdT. In contrast, patients with lower IL-38 and AST levels likely indicated a weak endogenous clearance of HBV infection. NAs such as LdT may not show potent antiviral efficacy without a strong endogenous clearance.

PNR suggests failure of antiviral treatment. Thus, in a compliant patient with PNR, more potent antiviral therapy might be necessary[21]. At 12 wk of LdT therapy, serum IL-38 levels of patients with PNR were lower than those of patients with PR, suggesting that patients with high IL-38 levels at baseline show a more effective clearance of HBV infection, which supports our speculation of more vigorous endogenous viral clearance in patients with a high serum level of IL-38.

Previous studies indicated that cytokines were involved in the noncytopathic suppression of HBV replication[23]. IL-6 is reported to be responsible for early suppression of HBV in infected hepatocytes[24]. IL-6 exerted its inhibitory effect through reducing HBV transcripts/core protein and decreasing the level of HBV genome-containing nucleocapsids[25]. IL-6 also inhibits HBV entry through down regulation of sodium taurocholate cotransporting polypeptide[26]. IL-12 plays an important role in the defense against viral infections through promoting naive T cells differentiation into Th1 cells and inhibiting viral replication[27]. Cytokine IL-12 can rescue the anti-viral function of exhausted HBV-specific CD8 T cells[28]. Our data showed positive correlations between IL-38 and IL-6 or IL-12, suggesting the functional association between cytokine IL-38 and Th1/Th2 type cytokines IL-6 and IL-12. Further studies are required to uncover the underlying molecular links between these cytokines.

Accurate prediction of response to antivirals in treating chronic HBV infection remains challenging. However, our findings strongly suggest that a favorable response does not completely depend on the antiviral efficacy of the agent, but also relies on the vigorous endogenous viral clearance. Thus, our study provides a clue for predicting VR by looking for signs of endogenous viral clearance.

In conclusion, our study demonstrated that higher pretreatment serum IL-38 levels are associated with a greater probability of VR to LdT treatment. Elevated levels of serum IL-38 in untreated patients with CHB reflect ongoing liver injury, which is an indirect indicator of vigorous endogenous clearance of HBV infection. A favorable response may be observed if the LdT antiviral effect is combined with a strong endogenous viral clearance. Therefore, our findings suggest that clear signs of viral clearance at baseline may predict a favorable response to treatment of CHB using NAs.

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COMMENTS

Background

Hepatitis B virus (HBV) infection is a serious global health concern. Interleukin (IL)-38 is a new anti-inflammatory cytokine of the IL-1 family, and its polymorphisms are associated with inflammatory diseases.

Research frontiers

The clinical outcome of HBV infection is a complex process between viral replication and host immune response. Cytokines are involved in the regulation of immune responses against HBV infection. Many investigators attempt to find ideal immunological indicators for response to antiviral therapy of chronic hepatitis B (CHB).

Innovations and breakthroughs

To date, there are no available data regarding the expression or function of IL-38 in CHB. This study demonstrated that higher serum IL-38 levels at pretreatment were associated with a greater probability of VR to LdT treatment. Elevated levels of serum IL-38 in untreated patients with CHB reflect ongoing liver injury, which is an indirect indicator of vigorous endogenous clearance of HBV infection. A favorable response could be observed if the LdT antiviral effect is combined with a strong endogenous viral clearance.

Applications

The findings suggest that clear signs of viral clearance at baseline may predict a favorable response to NAs treatment of CHB.

Peer-review

This manuscript demonstrated that higher pretreatment serum IL-38 levels are associated with a greater probability of VR to LdT treatment. The authors suggested that elevated serum IL-38 levels reflect ongoing liver injury, which is an indicator of endogenous clearance of HBV infection. These findings are interesting because it might provide novel predictors for good response to antiviral therapy.

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ORIGINAL ARTICLE

Basic Study

Expression of B7-H4 and hepatitis B virus X in hepatitis B virus-related hepatocellular carcinoma

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Author contributions: Hong B worked on the acquisition, analysis, and interpretation of data; Zhang H, Sang YW, and Wang Q carried out the cell experiments; Qian Y and Cheng LF carried out the IHC staining experiments; Zheng M and Yao HP designed the study and drafted the manuscript; all authors have read and approved the final manuscript.

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Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at yaohangping@zju.edu.cn. Participants gave informed consent for data sharing. No additional data are available.

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Abstract

AIM: To investigate the expression and clinical significance of B7-H4 and hepatitis B virus X (HBx) protein in hepatitis B virus-related hepatocellular carcinoma (HBV-HCC).

METHODS: The expression of B7-H4 in the human

HCC cell lines HepG2 and HepG2.2.15 were detected by western blot, flow cytometry, and immunofluorescence. The expression of B7-H4 and HBx in 83 HBV-HCC was detected by immunohistochemistry, and the relationship with clinicopathological features was analyzed. Paraffin sections were generated from 83 HBV-HCC patients (22 females and 61 males) enrolled in this study. The age of these patients ranged from 35 to 77 years, with an average of 52.5 ± 11.3 years. All experiments were approved by the Ethics Committees of the Second Affiliated Hospital, Zhejiang University School of Medicine.

RESULTS: B7-H4 was significantly upregulated in HepG2.2.15 cells compared to HepG2 cells. Specifically, the protein expression of B7-H4 in the lysates of HepG2 cells was more than that in HepG2.2.15 cells. In addition, HBx was expressed only in HepG2.2.15 cells. Similar data were obtained by flow cytometry. The positive rates of B7-H4 and HBx in the tissues of 83 HBV-HCC patients were 68.67% (57/83) and 59.04% (49/83), respectively. The expression of HBx was correlated with tumor node metastases (TNM) stage, and the expression of B7-H4 was positively correlated with HBx (r_s = 0.388; P < 0.01). The expression level of B7-H4 in HBx-positive HBV-HCC tissues was substantially higher than that in HBx-negative HBV-HCC tissues. The expression level of B7H4 was negatively related to tumor TNM stage.

CONCLUSION: Higher expression of HBx and B7-H4 was correlated with tumor progression of HBV-HCC, suggesting that B7-H4 may be involved in facilitating HBV-related hepatocarcinogenesis.

Key words: Hepatocellular carcinoma; Hepatitis B virus; Hepatitis B virus X; B7-H4; Immunohistochemistry

Core tip: Hepatitis B virus (HBV) is a major public health problem, and HBV-related hepatocellular carcinoma (HBV-HCC) has an extremely poor prognosis due to the lack of effective treatments. B7-H4 is a newly characterized member of the B7 superfamily that is actively involved in regulating the pathogenesis of tumors. However, the intrahepatic expression of B7-H4 in HBV-HCC patients has not been described. In this study, we found that the higher expression of HBx and B7-H4 was correlated with tumor progression of HBV-HCC. Therefore, B7-H4 may be involved in facilitating HBV-related hepatocarcinogenesis.

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INTRODUCTION

More than 350 million people worldwide suffer from a persistent hepatitis B virus (HBV) infection and are at an increased risk of developing hepatitis, cirrhosis, and hepatocellular carcinoma (HCC)[1]. HCC is the fifth most common cancer and the third most common cause of death due to cancer worldwide[2]. No systemic therapy has been shown to improve survival, and recurrence is common, even for curative treatment. Therefore, there is a need to identify prognostic biomarkers and new therapeutic strategies. It has been shown that the majority of HBV-related HCC tissue samples contain integrated viral DNA[3]. Hepatitis B virus X (HBx) originates from the HBV genome and is a multifunctional regulatory protein. Although HBx does not bind directly to DNA, it can *trans*-activate gene transcription through multiple *cis*-acting elements[4,5]. HBx may be associated with the development of human HCC[6,7], but the precise mechanism of HBx in tumorigenic transformation of hepatocytes remains unclear.

The coregulatory molecules of the B7 family have an indispensable role in the immune regulation of several pathologies[8]. It has been reported that B7-H1 and B7-H4 are expressed in human tumors and that they may act as coregulatory inhibitors to inhibit T cell activation or to induce T cell apoptosis[9-11]. Furthermore, the expression of B7-H1 in hepatocarcinoma cells can be initiated by HBx, leading to T cell apoptosis and potentially facilitating the genesis of HCC[12].

B7-H4 (also named B7S1 or B7x), a member of the B7 superfamily[13,14], has been demonstrated to inhibit T cell activation, proliferation, and differentiation and to be inversely correlated with tumor T cell infiltration[15,16]. B7-H4 is widely prevalent in a variety of tumor tissues[10,17-21], but it is absent on the cell surface of most human normal somatic tissues[13]. Multiple studies have revealed that B7-H4 can downregulate tumor-reactive cytotoxic T lymphocyte (CTL) function[13] and that inhibition of B7-H4 with small interference RNA (siRNA) led to antitumor immunity and inhibition of tumor growth[17,22]. Therefore, B7-H4 has been suggested to play a key role in tumor progression and immune escape. Interestingly, recent data have demonstrated that B7-H4 expression was enhanced in cells infected with a virus, such as Epstein-Barr virus (EBV)[23] and HBV[24]. These results indicate that B7-H4 signaling may affect the pathogenesis of a viral infection, and a clear understanding of its functional role may further elucidate the disease process. Although the presence of B7-H4 in human tumors appears to be a general phenomenon, there are no clinical data available on the expression levels of B7-H4 in human HBV-HCC.

It is evident that both HBx and B7-H4 are involved in the pathogenesis of HBV-HCC. However, the relationship between these two proteins remains unknown. Therefore, we speculated that both HBx and B7-H4 would promote the survival of hepatocytes transfected with HBV malignant transformation and tumor development. To test this hypothesis, HepG2 cells, a human HCC cell line, were transfected with HBV (HepG2.2.15). We investigated the prognostic significance and clinical relevance of HBx and B7-H4 expression in a cohort of 83 HCC patients treated by a curative resection.

MATERIALS AND METHODS

Cell lines

HepG2 cells derived from human HCC were purchased from American Type Culture Collection (ATCC, Manassas, VA, United States) and were grown in Dulbecco's Modified Eagle' Medium (DMEM) culture media (Life Technologies Corporation, Gaithersburg, MD, United States) containing 100 IU/mL penicillin, 100 μg/mL streptomycin (Life Technologies Corporation), and 10% fetal bovine serum (FBS, PAA, Morningside, Australia) at 37 ℃ in a humidified incubator with 5% CO_2.

HepG2.2.15 cells transfected with the complete HBV DNA and stably HBV-expressing hepatoma cells derived from HepG2 cells (firstly described by Sells et at 1987 and 1988)[25,26] were purchased from the Chinese Center For Type Culture Collection (CCTCC, Wuhan, China) and were grown in DMEM culture media containing 300 μg/mL G418 (Promega, Madison, WI, United States), 100 IU/mL penicillin, 100 μg/mL streptomycin, and 10% FBS at 37 ℃ in a humidified incubator with 5% CO_2.

Antibodies

Mouse monoclonal antibody (3E8 mAb) specific for human B7-H4 was previously described[27]. Rabbit anti-HBx antibody was purchased from Abcam (Cambridge, MA, United States). Horseradish peroxidase (HRP)-conjugated rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody was purchased from Cell Signaling Technology (Danvers, MA, United States). HRP-conjugated goat anti-mouse IgM, goat anti-mouse IgG, and goat anti-rabbit IgG antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, United States). Alexa Fluor® 488 goat anti-mouse IgM antibody was purchased from Invitrogen (Life Technologies Corporation).

Cell lysis

Cells were lysed using cell lysis buffer containing 2 mmol/L Tris-HCl, pH 7.5, 15 mmol/L NaCl, 0.1 mmol/L Na_2EDTA, 0.1 mmol/L EGTA, 0.1% Triton X-100, 0.25 mmol/L sodium pyrophosphate, 0.1 mmol/L beta-glycerophosphate, 0.1 mmol/L Na_3VO_4, and 0.1 μg/mL leupeptin (Cell Signaling Technology). Samples containing 1 × 10^6 cells were lysed in 200 μL cell lysis buffer for 30 min at 4 ℃ and then clarified by centrifugation at 8000 *g* for 10 min. Protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, United States), with bovine serum albumin (BSA) as the standard.

Western blot analysis

Cell lysate preparation and western blot were performed as described previously[27]. Briefly, cell lysates were denatured for 10 min at 95 ℃ with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, electrophoresed on 10% SDS-PAGE gels, and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% nonfat milk in Tris-buffered saline with Tween [TBST 20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, and 0.05% (v/v) Tween 20] and then incubated with specific antibodies at 4 ℃ overnight. After thoroughly washing, blots were incubated with HRP-conjugated secondary antibody for 1 h at room temperature. Protein band intensity was analyzed using enhanced chemiluminescence (ECL) reagents (Millipore, Billerica, MA, United States) and a VersaDoc MP5000 imaging system (Bio-Rad, Hercules, CA, United States).

Flow cytometry analysis

Each sample of cells (2 × 10^6) was incubated with 3E8 mAb for 1 h at 4 ℃, followed with Alexa Fluor® 488 goat anti-mouse IgM antibody for 30 min at 4 ℃. Normal mouse IgM was used as an antibody control. Cells were washed twice, and samples were analyzed using a flow cytometer (FACScan, San Jose, CA, United States) by flowjo7.6.

Immunofluorescence detection

Cell samples were fixed in ice-cold 3%-4% paraformaldehyde in phosphate buffered saline (PBS) (pH 7.4) for 15 min at room temperature, incubated for 10 min with PBS containing 0.25% Triton X-100, and then incubated with 1% BSA in PBS with Tween (PBST) for 30 min. Cells were then incubated in the diluted 3E8 mAb in 1% BSA in PBST in a humidified chamber overnight at 4 ℃. Normal mouse IgM was used as an antibody control. After thorough washing, cells were incubated with Alexa Fluor® 488 goat anti-mouse IgM antibody in 1% BSA for 1 h at room temperature in the dark and then rinsed with PBS. Cells were incubated with 3 ng/mL 4',6-diamidino-2-phenylindole (DAPI, Invitrogen, Life Technologies Corporation) for 10 min, mounted with ProLong Gold Antifade Reagent (Invitrogen, Life Technologies Corporation), and observed under an Olympus 1X81 fluorescence microscope (Center Valley, PA, United States) microscopy using filters for fluorescein isothiocyanate (FITC) and DAPI.

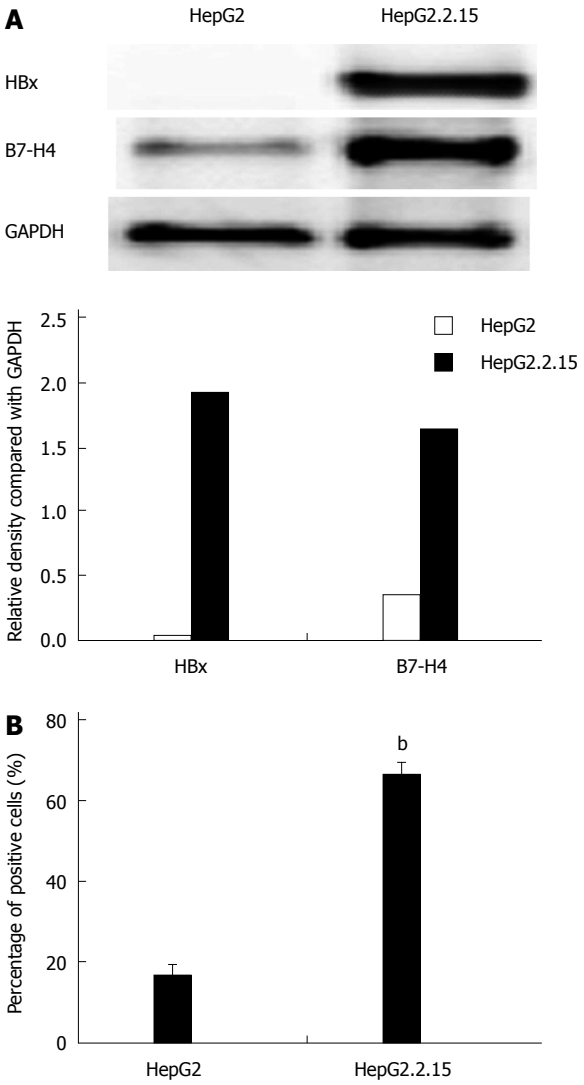

Figure 1 Expression of B7-H4 in HepG2 and HepG2.2.15 cells. A: Detection of B7-H4 expression by western blot. The expression level of B7-H4 and HBx was shown by the relative density ratio of B7-H4 and GAPDH; B: Detection of B7-H4 expression by flow cytometry. $^{b}P < 0.01$, HepG2.2.15 *vs* HepG2 cells. HBx: Hepatitis B virus X; GAPDH: Reduced glyceraldehyde-phosphate dehydrogenase.

Immunohistochemical staining

HBV-HCC tissue microarrays were obtained from Alenabio Biotechnology Co., LTD (Shanxi, China) for immunohistochemical (IHC) staining. Clinical and pathological information for individual cancer samples was provided by the array manufacturers (for details see www.alenabio.com). Paraffin sections of tumors were obtained from 83 HBV-HCC patients (22 females and 61 males) enrolled in this study. Informed consent for this study was obtained from each patient. The age of these patients ranged from 35 to 77 years, with an average of 52.5 ± 11.3 years. Tumor grade was divided into three categories: tumor grade Ⅰ is well-differentiated, low grade malignant; tumor grade Ⅱ is moderately-differentiated, intermediate grade malignant; tumor grade Ⅲ is poorly-differentiated, high grade malignant. TNM stage refers to the Tumor Node Metastasis stage. All of these experiments were approved by the Ethics Committees of the Second Affiliated Hospital, Zhejiang University School of Medicine. IHC staining was performed on mouse tumor tissues as previously described[27]. Briefly, tissues were labeled using primary antibodies specific for B7-H4 and HBx, and antibody binding was detected using EnVision System reagents (DAKO, Glostrup, Denmark). Semi-quantitative measurements of staining intensity (0-3, least intense to most intense), and the proportion of stained cells (0-4, no cells stained to more than 70% cells stained) were determined as previously described[27]. A combined score of $\geqslant 2$ was considered to indicate positive expression. All slides were scored by two researchers blinded to the pathological and clinical features.

Statistical analysis

All experiments were performed in triplicate. Differences in positive rates between groups were determined using a χ^2 test (SPSS 18, IBM Corporation, New York, NY, United States). Differences of B7-H4 expression between groups were determined using a Student's *t*-test. A correlation analysis was determined using Spearman correlation coefficients. Statistical significance was set at $P < 0.05$.

RESULTS

Detection of B7-H4 expression by Western blot and flow cytometry

The expression of B7-H4 in lysates of HepG2 and HepG2.2.15 cells was determined by western blot (Figure 1A). The results revealed that HBx was expressed in HepG2.2.15 cells but not in HepG2 cells. Moreover, the expression level of B7-H4 was significantly higher in HepG2.2.15 cells compared with HepG2 cells, consistent with results from the flow cytometry analysis ($P < 0.01$, Figure 1B).

Detection of B7-H4 expression by immunofluorescence staining

The immunofluorescence assay confirmed that the immunoreactivity for B7-H4 was strong in HepG2.2.15 cells. In contrast, the immunoreactivity for B7-H4 was virtually absent in HepG2 cells (Figure 2). Moreover, B7-H4 was diffusely expressed either in the membrane or the cytoplasm of HepG2.2.15 cells.

Expression and clinical significance of B7-H4 and HBx in HBV-related hepatocellular carcinoma

Immunohistochemical analysis detected the expression of B7-H4 and HBx in HBV-HCC tissues. Among the 83 HBV-HCC tissue samples examined, the positive rates of B7-H4 and HBx were 68.67% (57/83) and 59.04% (49/83), respectively. Statistical analysis revealed that the positive rates of HBx were correlated with the tumor TNM stage ($P < 0.01$) but not with

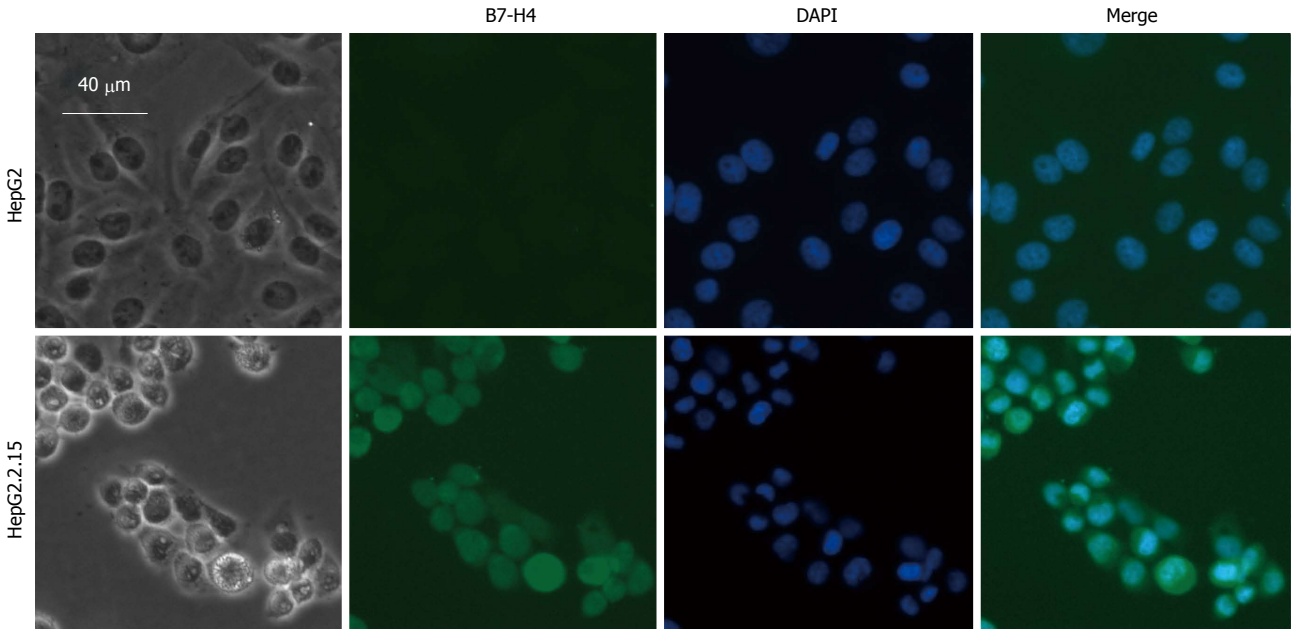

Figure 2 Expression of B7-H4 in HepG2 and HepG2.2.15 cells determined by immunofluorescence staining. Immunofluorescence detection of B7-H4 in HepG2 and HepG2.2.15 cells was stained with 3E8 mAb followed with Alexa Fluor® 488 goat anti-mouse IgM antibody (green). Nuclei were stained with DAPI (blue). Changes in cell morphology were recorded with a light microscope at 200 × magnification.

Table 1 Expression and clinical significance of B7-H4 and hepatitis B virus X in hepatitis B virus-related hepatocellular carcinoma

	HBx		χ^2/P	B7-H4		χ^2/P
	No. of positive	No. of negative		No. of positive	No. of negative	
Gender						
Male	39	22	$\chi^2 = 2.28$	42	19	$\chi^2 = 0.00$
Female	10	12	$P > 0.05$	15	7	$P > 0.05$
Age (yr)						
≤ 50	21	21	$\chi^2 = 2.87$	25	17	$\chi^2 = 3.31$
> 50	28	13	$P > 0.05$	32	9	$P > 0.05$
Tumor grade						
Ⅰ / Ⅱ	36	24	$\chi^2 = 0.08$	39	21	$\chi^2 = 1.36$
Ⅲ	13	10	$P > 0.05$	18	5	$P > 0.05$
TNM stage						
Ⅰ / Ⅱ	15	23	$\chi^2 = 11.09$	23	15	$\chi^2 = 2.16$
Ⅲ / Ⅳ	34	11	$P < 0.01$	34	11	$P > 0.05$

HBx: Hepatitis B virus X; HBV: Hepatitis B virus.

patient's age, sex, or tumor grade. The positive rate of HBx expression in TNM stage Ⅲ/Ⅳ tissues was 75.56% (34/45), substantially higher than the 39.47% in TNM stage Ⅰ/Ⅱ tissues (15/38, $P < 0.01$; Table 1). The positive rates of B7-H4 expression in HBV-HCC tissues were not correlated with patient's age, sex, tumor grade, or tumor TNM stage.

Correlation between B7-H4 and HBx in HBV-related hepatocellular carcinoma

The positive rate of B7-H4 in HBx-positive HBV-HCC tissues 83.67% (41/49) was significantly higher than the 47.06% in HBx-negative HBV-HCC tissues (16/34, $P < 0.01$). The expression of B7-H4 was positively correlated with HBx ($r_s = 0.388$, $P < 0.01$).

Furthermore, the effect of HBx on the expression level of B7-H4 in HBV-HCC tissues was analyzed (Table 2). B7-H4 expression with scores of 3.43 ± 1.68 in HBx-positive HBV-HCC tissues was substantially higher than that in HBx-negative HBV-HCC tissue scores of 1.81 ± 1.30 ($P < 0.01$). The expression level of B7-H4 in HBx-positive HBV-HCC tissues was not related to patient's age, sex, or tumor grade. However, a slight decrease was seen in TNM Ⅲ/Ⅳ samples (3.12 ± 1.63), and this difference was statistically significant in comparison with that of the TNM Ⅰ/Ⅱ samples (4.13 ± 1.51, $P < 0.05$; Figure 3).

DISCUSSION

HBV infection is the primary cause of liver disease that has the potential to develop HCC[3,28]. Although the

Table 2 Expression and clinical significance of B7-H4 in hepatitis B virus X-positive hepatitis B virus X-related hepatocellular carcinoma

	HBx-positive		*P* value
	B7-H4 expression scores	Case No.	
Gender			
Male	3.28 ± 1.65	39	> 0.05
Female	4.00 ± 1.56	10	
Age (yr)			
≤ 50	3.14 ± 1.62	21	> 0.05
> 50	3.64 ± 1.66	28	
Tumor grade			
Ⅰ / Ⅱ	3.33 ± 1.67	36	> 0.05
Ⅲ	3.69 ± 1.60	13	
TNM stage			
Ⅰ / Ⅱ	4.13 ± 1.51	15	< 0.05
Ⅲ / Ⅳ	3.12 ± 1.63	34	

HBx: Hepatitis B virus X; HBV: Hepatitis B virus.

pathogenic mechanisms of HBV-HCC are not clear, it has been reported in many studies that immune-mediated hepatocyte malignant transformation might play a key role. HCC has poor immunogenicity, partly due to poor antigen expression, the lack of costimulatory molecules to positively regulate T cell immune responses[29], and an increased number of costimulatory molecules that negatively regulate T cell immune response[30].

There is accumulating evidence that immunotherapy may become a powerful therapeutic option for HCC patients[31]. Stimulating or eliminating inhibitory T cell signaling to enforce antitumor responses is central to immune-based therapies to eradicate human cancers. It is clear that costimulatory molecules play pivotal roles in regulating the immune response and are engaged in the pathogenesis of many diseases, especially chronic virus infection and tumor development[32]. B7-H4 is a member of the B7 family, and its mRNA is broadly expressed in many tissues[13,14]. In addition to the inhibition of T cell tolerance, our previous data showed that B7-H4 was also involved in the regulation of cellular tumor apoptosis[22]. More importantly, after performing a multivariate adjustment for conventional prognostic factors, elevation in B7-H4 expression was a significant predictor of tumor recurrence[33]. The expression and distribution of B7-H4 in HCC tissues from HBV patients has not been previously reported. In this study, we found that the expression of B7-H4 was significantly increased in HCC cells transfected with HBV compared with control HCC cells. A high percentage of B7-H4 positive staining was observed in a subset of HBx-positive HBV-HCC tissues and was significantly associated with the TNM stage of HCC and level of HBx expression. Thus, HBx and B7-H4 may jointly promote the development of HBV-HCC and prove useful for the clinical evaluation of HBV-HCC patients, especially to identify high-risk patients who are at increased risk of cancer progression.

Although several studies have examined the role of B7-H4 in tumor immunity, the pathophysiologic function of B7-H4 has yet to be fully elucidated. In the present study, we found a detrimental role for B7-H4 in HBV-HCC patients. Costimulatory molecules, including vascular cell adhesion molecule (VCAM)-1[34], cluster of differentiation (CD)40[35], CD28, and programmed death (PD)-1[36] as well as several members of the B7 superfamily (*e.g.*, B7-1, B7-2[29], B7-H1[12,30], and B7-H3[37]), have been reported to be expressed in HCC tissues. These costimulatory molecules could provide positive (VCAM-1, CD28, CD40, B7-1, and B7-2) or negative (PD-1 and B7-H1) signals to local T cells response and regulate the pathogenesis of HCC. It has been reported that HBeAg suppresses the specific cellular immunity that clears the virus by upregulating B7-H1 expression, eventually leading to immune tolerance to HBV infection[38]. B7-H4 was described to be a membrane costimulatory ligand of the B7 superfamily, which is involved in the downregulation of T cell activation under certain circumstances. Ectopic B7-H4-Ig may protect animals from liver injury induced by concanavalin A (ConA), which could be associated with reduced serum levels of interleukin (IL)-2, interferon (IFN)-gamma, and IL-4 as well as enhanced IL-10 production[39]. Thus, B7-H4 may lead to immune tolerance to HBV infection and play an important role in immune suppression in chronic HBV-HCC patients. In addition to surface location, intracellular expression of B7-H4 has been reported in primary ovarian tumor cells[9], with similar expression levels to what we observed here in HBV-HCC (Figures 2 and 3). However, we also found that B7-H4 was an intracellular protein that was upregulated in HBV-HCC patients. This expression pattern suggests that B7-H4 might possess functions that are different from other cell surface molecules of the B7 family, which were previously reported to a play role in the pathogenesis of cancers by inhibiting the T cell-mediated immune response. Our previous results indicated that intracellular B7-H4 enhanced oncogenicity and inhibited apoptosis in pancreatic cancer cells[22]. In addition, Salceda *et al*[17] suggested that the expression of B7-H4 on breast cancer cell surface acts as an anti-apoptosis molecule that inhibits tumor apoptosis and ultimately protects tumors from cell-mediated immune surveillance. Furthermore, B7-H4 expression was enhanced in B cells infected with EBV, and the engagement of B7-H4 initially increased the levels of intracellular ROS, which induced the expression of FasL and subsequently aroused Fas-mediated and caspase-dependent apoptosis of EBV-transformed B cells[23]. These results together with our previous data indicate that B7-H4 signaling is involved in regulating cell apoptosis and that intracellular B7-H4 might possess an anti-apoptotic effect in HCC cells. Additional research is needed to determine if this effect is a function of B7-H4.

Interestingly, the expression of B7-H4 in HBx-

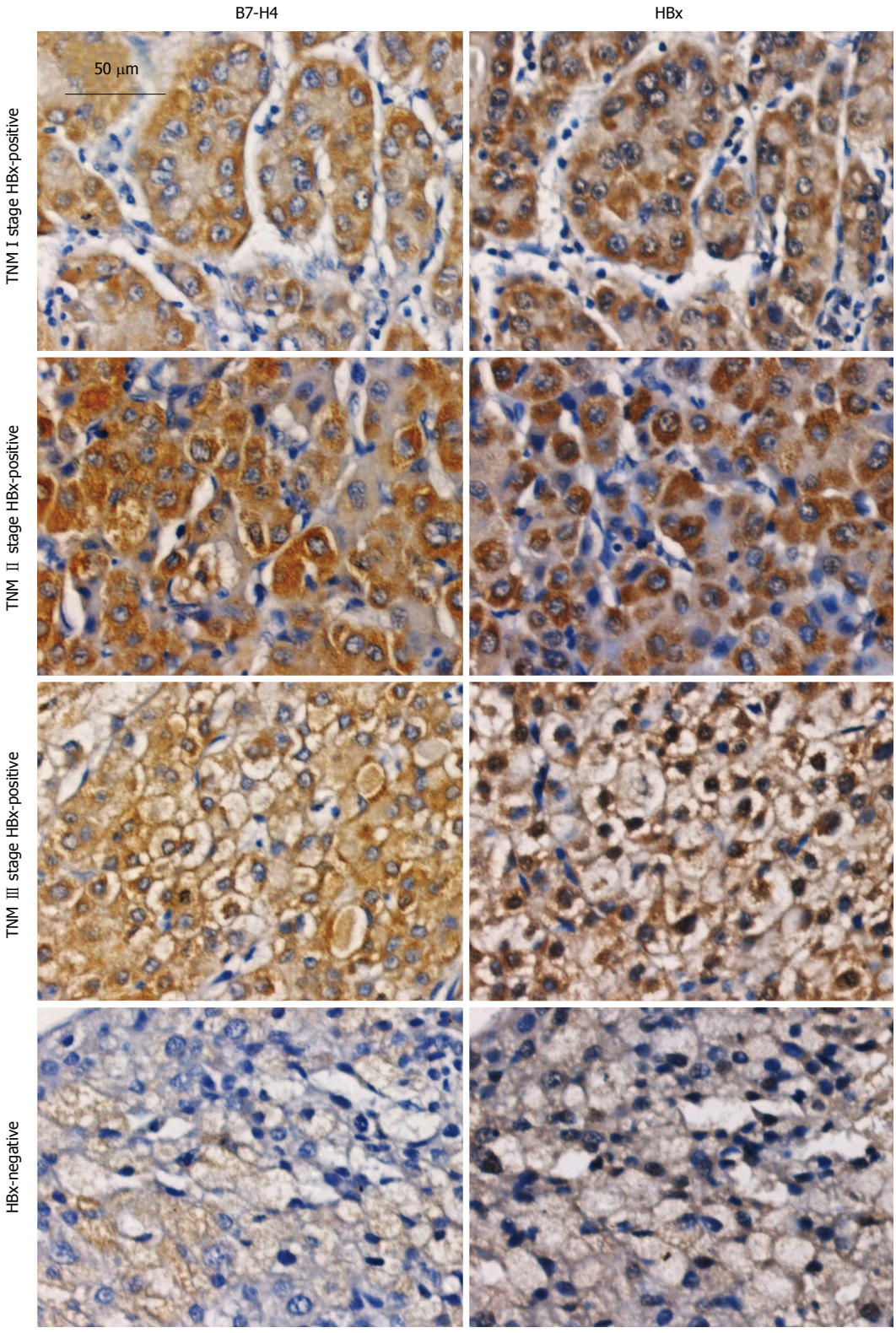

Figure 3 Expression of B7-H4 and hepatitis B virus X in hepatitis B virus related hepatocellular carcinoma tissues detected by immunohistochemical staining. Paraffin sections were generated from 83 hepatitis B virus related hepatocellular carcinoma (HBV-HCC) patients (22 females and 61 males) were enrolled in this study. The age of these patients ranged from 35 to 77 years, with an average of 52.5 ± 11.3 years. HBV-HCC tissues were subjected to IHC staining using antibodies specific for B7-H4 and HBx and recorded with a light microscope at 200 × magnification. HBx: Hepatitis B virus X; IHC: Immunohistochemical.

positive HBV-HCC tissues was negatively correlated with the TNM stage. The epitope binding to the 3E8 mAb of B7-H4 may be altered. It has been reported that some cell surface adhesive proteins, such as mucin 4 (MUC4), can mask the human epidermal growth factor receptor (HER)2 receptor and inhibit

Herceptin binding to HER2 through a stereospecific blockade, leading to Herceptin resistance[40]. Genetic mutations of epidermal growth factor receptor lead to gefitinib resistance[41]. Thus, we suspected that an epitope change of B7-H4 might be involved in immune-mediated tumor escape, thereby contributing to tumor development. Moreover, there was extensive necrosis in the advanced stage HBV-HCC tissues, leading to a decrease in the expression level of B7-H4. The mechanism by which this process occurs will be persued as an anvenue of future research.

In conclusion, our results indicate that B7-H4 is highly expressed in human HBx-positive HBV-HCC tissues and is associated with the TNM stage. Therefore, HBx and B7-H4 may play important roles in the development of HBV-HCC. In addition, B7-H4 may be involved in HBx-induced hepatocarcinogenesis, and an increased understanding of the functional roles of B7-H4 could aid in clarifying the mechanism of HBx in HBV-HCC. Furthermore, targeting B7-H4 may present a novel strategy by which disease diagnosis or immunotherapy against HBV infection can be achieved.

COMMENTS

Background

Hepatitis B virus-related hepatocellular carcinoma (HBV-HCC) has an extremely poor prognosis due to the lack of effective treatments. B7-H4 is a newly characterized member of the B7 superfamily of proteins, which are actively involved in regulating the pathogenesis of tumors. It is clear that both hepatitis B virus X (HBx) and B7-H4 are involved in the pathogenesis of HBV-HCC. However, the intrahepatic expression of B7-H4 in HBV-HCC patients has not been described.

Research frontiers

HBx originates from the HBV genome and is a multifunctional regulatory protein. Although HBx does not bind directly to DNA, it can *trans*-activate gene transcription through multiple *cis*-acting elements. HBx was thought to be associated with the development of human HCC. It has been reported that B7-H4 is expressed in human tumors and is implicated as coregulatory inhibitors that may inhibit T cell activation or induce T cell apoptosis upon antigen recognition. B7-H4 expression was enhanced in cells infected with a virus, such as Epstein-Barr virus (EBV) and HBV. B7-H4 signaling is most likely to affect the pathogenesis of a viral infection and a clear understanding of its functional role may further elucidate the disease process. Although the presence of B7-H4 in human tumors appears to be a general phenomenon, there is little available on the expression level of B7-H4 in human HBV-HCC.

Innovations and breakthroughs

In this article, the authors reached the conclusion that co-inhibitory molecules, such as B7-H4, are always involved in HBx-induced hepatocarcinogenesis. Targeting B7-H4 may represent a novel strategy by which disease diagnosis or immunotherapy against HBV infection can be achieved.

Applications

Based on these data, the authors concluded that B7-H4 is highly expressed in human HBx-positive HBV-HCC tissues and is associated with TNM stage. Therefore, HBx and B7-H4 may play an important role in the development of HBV-HCC. In addition, B7-H4 may be involved in HBx-induced hepatocarcinogenesis, and an increased understanding of the functional roles of B7-H4 could aid in clarifying the mechanism of HBx in HBV-HCC. This study provides new insight into the function of a coinhibitory signaling pathway during the clinical course of HBV-HCC.

Peer-review

The authors have presented a highly interesting study. The design, interpretation, and presentation of their study are well performed.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Predictive factors for survival and score application in liver retransplantation for hepatitis C recurrence

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Abstract

AIM: To identify risk factors associated with survival in patients retransplanted for hepatitis C virus (HCV) recurrence and to apply a survival score to this population.

METHODS: We retrospectively identified 108 patients retransplanted for HCV recurrence in eight European liver transplantation centers (seven in France, one in Spain). Data collection comprised clinical and laboratory variables, including virological and antiviral treatment data. We then analyzed the factors associated with survival in this population. A recently published score that predicts survival in retransplantation in patients with hepatitis C was applied. Because there are currently no uniform recommendations regarding selection of the best candidates for retransplantation in this setting, we also described the clinical characteristics of 164 patients not retransplanted, with F3, F4, or fibrosing cholestatic hepatitis (FCH) post-first graft presenting with hepatic decompensation.

RESULTS: Overall retransplantation patient survival rates were 55%, 47%, and 43% at 3, 5, and 10 years, respectively. Patients who were retransplanted for advanced cirrhosis had survival rates of 59%, 52%, and 49% at 3, 5, and 10 years, while those retransplanted for FCH had survival rates of 34%, 29%, and 11%, respectively. Under multivariate analysis, and adjusting for the center effect and the occurrence of FCH, factors associated with better survival after retransplantation were: negative HCV viremia before retransplantation, antiviral therapy after retransplantation, non-genotype 1, a Model for End-stage Liver Disease (MELD) score < 25 when replaced on the waiting list, and a retransplantation donor age < 60 years. Although the numbers were small, in the context of the new antivirals era, we showed that outcomes in patients who underwent retransplantation with undetectable HCV viremia did not depend on donor age and MELD score. The Andrés score was applied to 102 patients for whom all score variables were available, producing a mean score of 43.4 (SD = 6.6). Survival rates after the date of the first decompensation post-first liver transplantation (LT1) in the liver retransplantation (reLT) group (94 patients decompensated) at 3, 5, and 10 years were 62%, 59%, and 51%, respectively, among 78 retransplanted individuals with advanced cirrhosis, and 42%, 32%, and 16% among 16 retransplanted individuals with FCH. In the non-reLT group with hepatic decompensation, survival rates were 27%, 18%, and 9% at 3, 5, and 10 years, respectively (P < 0.0001). Compared with non-retransplanted patients, retransplanted patients were younger at LT1 (mean age 48 ± 8 years compared to 53 ± 9 years in the no reLT group, P < 0.0001), less likely to have human immunodeficiency virus (HIV) co-infection (4% *vs* 14% among no reLT patients, P = 0.005), more likely to have received corticosteroid bolus therapy after LT1 (25% in reLT *vs* 12% in the no reLT group, P = 0.01), and more likely to have presented with sustained virological response (SVR) after the first transplantation (20% in the reLT group *vs* 7% in the no reLT group, P = 0.028).

CONCLUSION: Antiviral therapy before and after retransplantation had a substantial impact on survival in the context of retransplantation for HCV recurrence, and with the new direct-acting antivirals now available, outcomes should be even better in the future.

Key words: Antivirals; Hepatitis C; Mortality; Prognosis; Retransplantation; Risk factors

Core tip: Liver retransplantation for hepatitis C recurrence may be a subject of debate. This study was performed in order to assist patient selection for retransplantation in a context of donor scarcity. This retrospective multicenter study analyzed predictive factors for survival in a population of patients retransplanted for hepatitis C virus recurrence, including virological and antiviral treatment data. We also applied a previously published score to this population.

Song ATW, Sobesky R, Vinaixa C, Dumortier J, Radenne S, Durand F, Calmus Y, Rousseau G, Latournerie M, Feray C, Delvart V, Roche B, Haim-Boukobza S, Roque-Afonso AM, Castaing D, Abdala E, D'Albuquerque LAC, Duclos-Vallée JC, Berenguer M, Samuel D. Predictive factors for survival and score application in liver retransplantation for hepatitis C recurrence. *World J Gastroenterol* 2016; 22(18): 4547-4558 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i18/4547.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i18.4547

INTRODUCTION

Advanced liver disease caused by hepatitis C virus (HCV) is the leading cause of liver transplantation (LT) in Western countries[1,2]. The post-transplant detection of HCV ribonucleic acid (RNA) in the serum or graft is universal in pre-LT viremic patients[3,4]. Histologically documented chronic hepatitis C develops in approximately 70% of patients during the first year after LT[5]. Progression of this disease is particularly aggressive in transplanted patients, with a rapid evolution towards fibrosis (cirrhosis within approximately 9 to 12 years) when compared to immunocompetent individuals (cirrhosis within approximately 20-30 years)[4], resulting in graft loss due to recurrent disease[6]. A previous study showed that patients with clinically compensated graft cirrhosis achieved a 1-year survival rate of 74%, but this rate fell to 41% in those with clinical decompensation[7]. In patients with established cirrhosis and graft failure, retransplantation (reLT) is the only therapeutic option[8]. However, because of organ shortages, cost issues, and poorer survival, the indications for reLT must be appropriate[9]. In previous studies, HCV-related disease did not indicate a poorer prognosis following reLT[9-21], while other studies identified HCV recurrence as an independent predictive factor of mortality[22-24]. Few studies have evaluated risk factors for mortality among patients retransplanted for HCV recurrence[25-29].

Predictive models for retransplantation

In order to predict post-reLT survival and, therefore, aid in patient selection, several scores have been developed[30,31]. That most widely employed is the Rosen score[32]. However, like many others, this model was based on reLT cases in general and not just on cases of HCV recurrence.

The first score specifically designed for HCV-positive patients was published recently by Andres *et al*[29]. This study analyzed registry data from the Scientific Registry of Transplant Recipients on 1422 individuals transplanted for HCV and retransplanted at least 30 d after the first transplant. In order to design a score that could predict survival after reLT, they identified six predictive variables associated with survival: recipient age at the time of the first transplant, interval elapsing between the two transplants, donor age, creatinine levels, international normalized ratio (INR), and serum albumin values before the second transplant.

Several factors currently influence the decision to retransplant patients who have experienced a recurrence of HCV after transplantation. These include factors related to the individual patient, the physician' s judgement, transplant cent policies and experience, and geographic donor organ availability. There are no uniform guidelines to indicate which patients with HCV recurrence should undergo reLT. A survey in 2003 showed that nearly all transplant centers in the United States were likely to offer reLT to patients experiencing an HCV recurrence[33]. The scenario is likely to change radically with the development of new direct-acting antivirals (DAA). However, these drugs may not be available in some countries in the short- or medium-term.

The present study was performed in order to identify predictive survival factors in patients with HCV-related graft failure undergoing liver reLT before the era of new antivirals and to apply a previously published score[29] that predicts survival after reLT in recipients with HCV recurrence. Secondary objectives were to describe the natural history of HCV in retransplanted patients compared to their first transplantation and to describe a group of patients experiencing graft failure due to HCV recurrence who were not retransplanted in order to clarify which patients were selected for reLT, as there are no uniform criteria for reLT in the setting of HCV recurrence.

MATERIALS AND METHODS

Study design and population

This was a retrospective and multicenter study involving seven liver transplantation centers in France (Paul Brousse, Edouard Herriot, Beaujon, Saint Antoine, Pitié-Salpétrière, Rennes and Henri Mondor Hospitals), and one center in Spain (La Fe Hospital). There were no specific recommendations regarding reLT criteria for HCV-related recurrence, and the indication for reLT depended on each center's policies. However, all centers generally indicated reLT in patients with graft failure, using the same criteria as those applied for the first transplantation. Protocol biopsies were performed yearly in all French centers, but not in the Spanish center. Immunosuppression protocols were similar, with the use of cyclosporine or tacrolimus, corticosteroids during the first 6-12 mo, and/or mycophenolate mofetil. Antiviral treatment policies were also similar; treatment was initiated with any degree of fibrosis or fibrosing cholestatic hepatitis, given that the clinical conditions were sufficient to undergo antiviral therapy.

We included patients aged 18 or older who had undergone LT for HCV-related disease and then reLT with HCV recurrence as the main indication between January 1994 and June 2012 (reLT group). HCV recurrence was confirmed histologically as the principal reason for reLT by prior biopsies or an explant displaying cirrhosis or fibrosing cholestatic hepatitis. Patients with hepatitis B (HBV) co-infection with positive HBV DNA after the first LT (LT1) were excluded.

In order to describe which patients were selected for reLT in the absence of uniform criteria for reLT in the setting of HCV recurrence, we also identified those transplanted for HCV-related disease and presenting with graft failure but who were not retransplanted (no reLT group). This group represented the population in whom reLT may have been indicated, as opposed to those with cirrhosis and no clinical decompensation, in whom reLT would not have been indicated. The

inclusion criteria were: patients aged 18 or older receiving LT between January 1994 and June 2012 for HCV-related disease and experiencing HCV recurrence in the form of Metavir F3, F4, or fibrosing cholestatic hepatitis (FCH) (confirmed histologically), presenting with clinical hepatic decompensation (defined as the presence of ascites, encephalopathy, variceal hemorrhage, or jaundice), who had not undergone reLT, and with positive serum HCV RNA after the first transplantation. Exclusion criteria were patients with HBV co-infection with positive HBV DNA after transplantation.

Case identification

Retransplanted patients were identified by consulting prospectively maintained databases in the Spanish center and in five of the French centers or the database operated by the French Biomedicine Agency (Agence de la Biomédecine), which covered the two other French centers. This agency is a public organization under the supervision of the French Ministry of Health, whose responsibilities include organizing the procurement and transplantation of organs, tissues, and cells. All transplant centers in France are required to report all their cases to this agency.

To identify cases of non-retransplanted patients with advanced liver disease/FCH and graft failure in all centers, all patients transplanted for HCV-related disease were identified first. Subsequently, all post-LT biopsies revealing F3, F4, or FCH were included, and a chart review was performed in order to identify those patients presenting with hepatic decompensation (defined as the presence of ascites, encephalopathy, variceal hemorrhage, or jaundice).

Data collection and definitions

After case identification, data were collected by consulting the prospectively maintained databases when available, which included several variables. Variables not available in the databases were collected through chart review in all centers.

Regarding the first and second transplantations: Donor age and gender, living or deceased donor, donor HCV serology, recipient age and gender, concomitant kidney transplantation, acute rejection episodes after LT, receipt of corticosteroid bolus, receipt of OKT3, maintenance immunosuppressive regimen, HCV treatment (medication and duration), reason for treatment discontinuation, HCV treatment response (as previously defined[34]), presence of hepatocellular carcinoma (HCC) prior to LT1, presence of alcoholic disease prior to LT1, human immunodeficiency virus (HIV) co-infection, diabetes mellitus pre or post-LT, Metavir fibrosis score on biopsies post-transplant, presence and timing of FCH, HCV viremia levels before LT1 and before reLT, HCV genotype, date and type of hepatic decompensation, biochemical data 20-30 d after decompensation, fibrosis progression rate[35], date and cause of death, reason for no reLT, and patient and graft survival (the latter being defined as the interval between the date of transplant and the date of hepatic decompensation or death).

Regarding the second transplantation: MELD score prior to transplant, pre-reLT bilirubin, pre-reLT creatinine, pre-reLT INR, pre-reLT albumin, date and type of decompensation, number of days of intensive care unit (ICU) hospitalization prior to reLT, date of graft failure (defined as clinical hepatic decompensation), and date and cause of death.

Survival score

The survival score published previously by Andres *et al*[29] was applied to all reLT cases for which such score variables were available. This score was calculated as follows:

(0.23 × donor age) + (4.86 × creatinine log) - (2.45 × interval between the first and second transplant log) + (2.69 × INR) - (0.10 × recipient age) + (3.27 × albumin + 40).

Statistical analysis

The primary endpoint was to determine predictive factors for survival after reLT for a recurrence of hepatitis C. Graft and patient survival probabilities were determined using the Kaplan-Meier method and compared using the log-rank test. A Cox model with a likelihood ratio test was used to compare the difference in survival for continuous variables. Variables with a *P* value below 0.15 under univariate analysis were included in order to enable a stepwise multivariate evaluation using the Cox multivariate model, with the calculation of hazard ratios and corresponding 95%CI. Under multivariate analysis, a *P* value of 0.05 or lower was considered to be significant. A predictive model was constructed with the aim of predicting survival in individual patients retransplanted for hepatitis C recurrence according to the presence of prognostic factors[36]. For this, donor age was categorized as more or less than 60 years considering the scarcity of young donors, and MELD score superior or inferior to 25[37]. Data were compared between retransplanted and non-retransplanted patients using the χ^2 test for categorical data and the independent samples *t*-test for continuous data. Survival comparisons between the two groups were performed by taking account of the date of the first decompensation, considering that this could be the moment at which re-listing would be discussed. Differences between fibrosis progression rates were calculated using a paired *t* test. A *P* value of 0.05 or lower was considered to be significant. Statistical analyses were performed using SAS software version 9.1.3 (SAS Institute Inc., Cary, NC, United States).

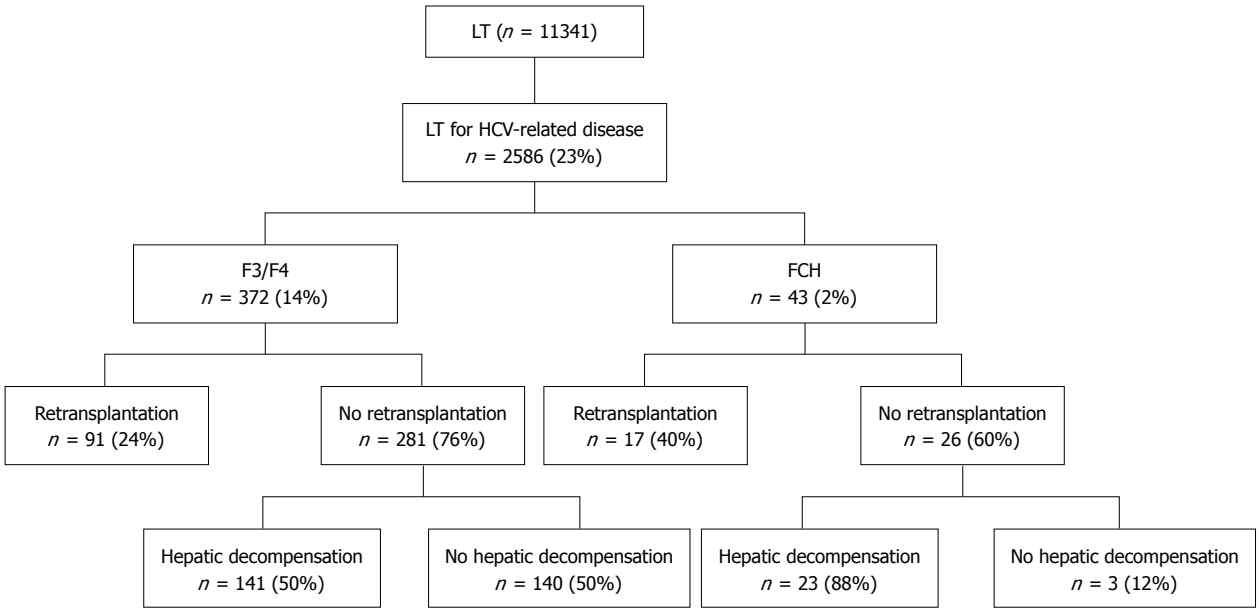

Figure 1 One hundred and eight retransplanted cases for hepatitis C virus recurrence and 164 not retransplanted F3/F4/ fibrosing cholestatic hepatitis cases presenting hepatic decompensation (in italics) after inclusion and exclusion criteria were applied. HCV: Hepatitis C virus; F3/F4: Metavir fibrosis score 3/4; FCH: Fibrosing cholestatic hepatitis.

RESULTS

Between January 1994 and June 2012, 11341 LTs were performed in the eight study centers, and in 2586 (23%) patients, the main indication was HCV-related disease. Of these, 372 (14%) patients progressed to F3 or F4, and 91 patients were retransplanted. Forty-three patients (2%) presented FCH, and 17 of these were retransplanted, totaling 108 retransplanted patients. Figure 1 shows all cases that led to the final case selection. We also identified 164 patients with hepatic decompensation who did not undergo reLT (141 with F3 or F4 and 23 with FCH) prior to the data collection period. The center-based distribution of advanced fibrosis and FCH cases with and without reLT is described in Table 1. The mean interval elapsing between reLT re-listing and actual reLT was 151 d (1-1393), with no statistical difference between the groups (P = 0.22). Explants revealed concomitant chronic rejection in three cases, non-alcoholic steatohepatitis in one case, and hepatocarcinoma in one case. In all other cases, HCV recurrence was the only diagnosis that led to reLT.

Demographic and clinical characteristics

The principal clinical and demographic characteristics of the reLT patients were: mean age at LT1 of 48 ± 8 years; mean age at reLT of 54 ± 8 years; 81 (75%) were men; mean donor age at LT1 of 51 ± 15 years; mean donor age at reLT of 44 ± 16 years; none of the donors were seropositive for HCV; concomitant alcoholic disease in 20 patients (18%); HIV co-infection in four (4%); HCC at LT1 in 40 (37%); 84 patients (78%) presented with F4 after LT1, 18 (17%) presented with FCH, and six (5%) presented with F3 and clinical hepatic decompensation; and 26 patients (25%) received corticosteroid bolus after LT1. The mean interval between LT1 and decompensation was 4.4 years (0.1-16.0). The mean interval between LT1 and reLT was 5.5 years (0.1-17.8). The mean MELD score 20-30 d after decompensation was 21 ± 7, and the mean MELD score before reLT was 24.5 ± 8.4. The first decompensation presented as ascites in 68 patients (68%), encephalopathy in 12 (12%), jaundice in 10 (10%), and variceal hemorrhage in 9 (9%).

Survival and prognostic factors

Patient survival rates in the reLT group were 55%, 47%, and 43% at 3, 5, and 10 years, respectively, after the date of reLT. Patients who were retransplanted for advanced cirrhosis had survival rates of 59%, 52%, and 49% at 3, 5, and 10 years, while those retransplanted for FCH had survival rates of 34%, 29%, and 11%, respectively.

There were 28 cases of reLT before 2003, and 80 cases between 2003 and 2012. There was no statistical difference when survival was compared between these two periods (P = 0.26).

Fourteen patients presented with a sustained virological response (SVR) after LT1. The survival rate in this group of patients was 86% at 5 years (12/14 patients), with a median reLT donor age of 44 years (14-62) and a median MELD score of 24 (14-38) (Table 2).

The risk factors associated with better survival under univariate analysis are shown in Tables 3 and 4. An Andrés score lower than 40 was significantly associated with better survival under univariate

Table 1 Distribution of hepatitis C virus-related cases of advanced fibrosis/fibrosing cholestatic hepatitis cases after liver transplantation with and without liver retransplantation according to center *n* (%)

Center	*n* (LT)	LT related to HCV (percent of total LT)	F3/F4/FCH after LT (percent of LT for HCV)	F3/F4/FCH reLT (percent of LT for HCV)	F3/F4/FCH without ReLT (percent of LT for HCV)
1	1933	770 (40)	174 (23)	31 (4)	143 (19)
2	2001	420 (21)	108 (26)	26 (6)	82 (20)
3	2124	412 (19)	20 (5)	12 (3)	8 (2)
4	1477	321 (22)	40 (12)	11 (3)	29 (9)
5	920	247 (27)	33 (13)	10 (4)	23 (9)
6	1576	141 (9)	16 (11)	8 (6)	8 (6)
7	450	143 (32)	16 (11)	8 (6)	8 (6)
8	860	132 (15)	8 (6)	2 (2)	6 (5)
Total	11341	2586 (23)	415 (16)	108 (4)	307 (12)

LT: Liver transplantation; reLT: Liver retransplantation; HCV: Hepatitis C virus; F3: Metavir score F3; F4: Metavir score F4; FCH: Fibrosing cholestatic hepatitis.

Table 2 Donor age, MELD score, and outcomes in patients undergoing liver retransplantation for hepatitis C virus recurrence with undetectable hepatitis C virus viremia before liver retransplantation

reLT donor age (yr)	reLT MELD score	Outcome
14	35	Alive
19	22	Deceased
20	24	Alive
23	16	Alive
24	25	Alive
31	17	Alive
35	23	Alive
46	33	Deceased
47	38	Alive
52	26	Alive
58	21	Alive
60	28	Alive
62	14	Alive

reLT: Liver retransplantation; MELD: Model for End-stage Liver Disease.

analysis but not under multivariate analysis. Factors associated with survival under multivariate analysis are shown in Table 5, which concerns the 83 patients for whom all variables found to be significant under univariate analysis were available. Data were adjusted for the center effect and for the occurrence of FCH after LT1.

The causes of death among the 55/108 patients (51%) in the reLT group were as follows: septic shock of bacterial origin in 17 (31%), liver failure due to HCV recurrence in nine (16%), surgical complications during the perioperative period in seven (13%), hemorrhage in five (9%), multiorgan failure in four (7%), heart failure in two (4%), septic shock of fungal origin in two (4%), and other causes in eight (16%) (one case each of lymphoproliferative disorder, pulmonary embolism, renal insufficiency, septic shock of mycobacterial origin, non-liver solid cancer, and four unknown).

Estimation of survival

An estimate of survival was calculated, based on the presence of the five independent predictors of survival (Table 6). This table highlights two situations that underline the importance of HCV therapy after reLT. If modifiable factors were taken into account and a donor younger than 60 years was used, with the patient receiving antiviral treatment after reLT, estimated survival at 3 years was 73%. In contrast, the same situation but with no treatment after reLT generated a survival rate of only 18% at 3 years.

Application of the score

The Andrés score was applied to the 102 patients for whom all score variables were available (in six patients, pre-reLT albumin values were not available), and this produced a mean score of 43.4 (SD = 6.6).

Natural history

In the reLT group, 18/108 patients (17%) presented with FCH after the first LT. Of these, 5/18 (28%) progressed to FCH after reLT (P = 0.11). Of the remaining 90/108 (83%) who did not present with FCH after LT1, six (7%) progressed to FCH after reLT.

In 52 patients with available pre and post-reLT biopsies, the mean fibrosis progression rate was 2.28 (0.27-16) Metavir units/year after LT1, compared to 1.49 (0-6.0) Metavir units/year after reLT (P = 0.051). Of these, 13 (25%) received antiviral therapy before reLT (four patients presented with an SVR), and 11 (21%) received antiviral therapy after reLT (seven presented with an SVR).

Fifty-six of the remaining patients were not included in the analysis for the fibrosis progression rate for the following reasons: five did not undergo biopsies before reLT (so that the precise timing of fibrosis was not determined), 51 did not present biopsies with fibrosis after reLT (23 died within 90 d of reLT, six underwent biopsies that revealed lobular hepatitis, five had biopsies showing FCH, five had undetectable levels of HCV viremia before reLT, two presented with a sustained virological response after antiviral therapy following reLT, and ten for unknown reasons).

Table 3 Univariate analysis of qualitative variables associated with survival in retransplanted patients for hepatitis C virus recurrence

Risk factor	*n*	Survival estimation			Log-rank *P* value
		3 yr	5 yr	10 yr	
HIV serology					
Negative	104	57%	49%	45%	0.006
Positive	4	0%			
IS after LT1					
Without MMF	82	58%	51%	47%	0.060
With MMF	26	45%	33%	0%	
Genotype 1					
No	23	69%	69%	69%	0.110
Yes	69	55%	45%	41%	
HCV viremia pre-reLT					
Negative	14	86%	86%	86%	0.005
Positive	94	50%	41%	36%	
Dialysis pre-reLT					
Yes	19	74%	67%	67%	0.060
No	89	51%	43%	37%	
Split graft at reLT					
No	101	57%	48%	44%	0.060
Yes	7	29%	29%	0%	
IS after reLT					
With tacrolimus	62	67%	57%	53%	0.038
Without tacrolimus	41	43%	37%	30%	
Antiviral therapy post-reLT					
Yes	35	85%	75%	64%	0.0003
No	63	44%	36%	36%	
Antiviral response post-reLT					
SVR	14	93%	93%	93%	0.039
Partial or NR	18	77%	59%	43%	
FCH post-LT1					
No	90	59%	52%	49%	0.018
Yes	18	34%	23%	0%	
Arterial complications post-reLT					0.030
No	90	62%	53%	48%	
Yes	11	27%	27%	27%	
Andres score > 40					
No	35	71%	64%	64%	0.019
Yes	67	49%	40%	37%	
Reinscription MELD < 25					0.026
No	30	39%	34%	34%	
Yes	72	66%	58%	51%	

HIV: Human immunodeficiency virus; IS: Immunosuppression; LT1: First liver transplantation; MMF: Mycophenolate mophetil; HCV: Hepatitis C virus; reLT: Liver retransplantation; SVR: Sustained virological response; NR: Non-responder; FCH: Fibrosing cholestatic hepatitis; MELD: Model for End-stage Liver Disease.

No reLT group

Survival rates after the date of the first decompensation post-LT1 in the reLT group (94 patients decompensated) at 3, 5, and 10 years, were 59%, 55%, and 46%, respectively. In the non-reLT group with hepatic decompensation, survival rates were 27%, 18%, and 9% at 3, 5, and 10 years, respectively ($P < 0.0001$).

Compared to non-retransplanted patients, retransplanted patients were younger at LT1 (mean age 48 ± 8 years compared to 53 ± 9 in the no reLT group, $P < 0.0001$), less likely to have HIV co-infection (4% compared to 14% in the no reLT group, $P = 0.005$), more likely to have received corticosteroid bolus after LT1 (25% in the reLT *vs* 12% in the no reLT group, $P = 0.01$), and more likely to have presented with an SVR after the first transplantation (20% in reLT group *vs* 7% in no reLT group, $P = 0.028$) (the data refer to 71 treatments in reLT group and 87 treatments in the no reLT group). Variables found to be similar in both groups included: acute rejection episodes after LT1, use of OKT3 after LT1, number of antiviral treatments, results of biochemical and hematological investigations up to 30 d after hepatic decompensation following LT1 (bilirubin, creatinine, INR, hemoglobin, platelets, sodium and albumin), genotype distribution, type of antiviral treatment, treatment duration, and rate of treatment discontinuation.

Table 4 Univariate analysis of quantitative variables associated with survival in retransplanted patients for hepatitis C virus recurrence

Risk factor	Hazard ratio	95%CI	*P* value
Less days under MV pre-reLT	1.25	1.02-1.52	0.031
Lower reLT donor age	1.02	1.00-1.04	0.017
Lower recipient age at LT1	1.04	1.00-1.08	0.027
Greater interval between LT1 and reLT	0.88	0.82-0.96	0.002

MV: Mechanical ventilation; reLT: Liver retransplantation; LT1: First liver transplantation.

Table 5 Factors associated with survival according to multivariate analysis in patients undergoing liver retransplantation for hepatitis C virus recurrence, adjusted for center effect and fibrosing cholestatic hepatitis occurrence

Risk factor	Hazard ratio	95%CI	*P* value
Undetectable HCV viremia pre-reLT	8.80	1.96-39.39	0.004
Receipt of antiviral treatment after reLT	4.98	2.23-11.15	< 0.0001
ReLT donor age < 60 yr	3.54	1.42-8.82	0.007
Non-genotype 1	3.94	1.35-11.57	0.010
Reinscription MELD ⩽ 25	2.45	1.23-4.88	0.010

HCV: Hepatitis C virus; reLT: Liver retransplantation; MELD: Model for End-stage Liver Disease.

In the non-reLT group with hepatic decompensation, 20 (12.1%) of the 164 patients were re-listed or were undergoing a pre-reLT work-up for relisting at the time of data collection. The reasons for not replacing the remaining 144 patients on the waiting list were: death due to hepatic decompensation before relisting (30.6%), clinically considered as unsuitable because of hepatic, cardiac, renal, neurological, psychiatric or other systemic diseases (22.9%), advanced age (over 70 years) (11.8%), *de novo* cancer or HCC recurrence (6.9%), alcohol consumption (4.9%), stable without further decompensation (4.2%), under antiviral therapy at the time of data collection and would be considered for reLT depending on outcome (2.4%), poor compliance (2.8%), stabilized after a SVR (1.4%),

Table 6 Survival estimation according to the presence of each of the independent mortality risk factors

RT inscription MELD > 25	Genotype 1	RT donor age > 60 yr	No antiviral treatment post-RT	HCV viremia pre-RT	Number of factors	Survival		
						1 yr	3 yr	5 yr
-	-	-	-	-	0	99.7%	99.5%	99.3%
+	-	-	-	-	1	99.1%	98.7%	98.3%
-	+	-	-	-		98.8%	98.1%	97.2%
-	-	+	-	-		99.0%	97.9%	97.0%
-	-	-	+	-		98.6%	97.7%	96.8%
-	-	-	-	+		98.0%	96.2%	94.8%
+	+	-	-	-	2	97.4%	95.3%	94.6%
+	-	+	-	-		97.7%	95.9%	94.3%
+	-	-	+	-		96.9%	95.1%	93.2%
-	+	+	-	-		96.0%	93.4%	90.8%
+	-	-	-	+		95.3%	91.7%	88.3%
-	+	-	+	-		94.8%	91.3%	87.9%
-	-	+	+	-		94.8%	90.7%	87.3%
-	+	-	-	+		91.3%	85.9%	81.0%
-	-	+	-	+		91.3%	85.7%	79.6%
-	-	-	+	+		87.0%	79.4%	71.2%
+	+	+	-	-	3	92.3%	87.8%	83.2%
+	+	-	+	-		89.4%	82.5%	76.4%
+	+	-	-	+		83.2%	73.1%[1]	63.9%
+	-	+	-	+		82.5%	72.1%	63.1%
-	+	+	+	-		82.0%	71.2%	61.9%
+	-	-	+	+		75.2%	61.4%	50.3%
+	-	+	+	-		72.5%	58.1%	47.0%
-	+	+	-	+		71.1%	56.3%	44.5%
-	+	-	+	+		61.2%	42.7%	30.2%
-	-	+	+	+		59.9%	41.6%	28.5%
+	+	+	+	-	4	67.2%	50.5%	37.9%
+	+	+	-	+		52.0%	32.4%	20.1%
+	+	-	+	+		36.9%	18.1%[1]	8.9%
+	-	+	+	+		35.7%	17.1%	8.3%
-	+	+	+	+		17.0%	4.5%	1.5%
+	+	+	+	+	5	2.8%	0.3%	0.0%

[1]Two situations that underline the importance of HCV therapy after reLT. HCV: Hepatitis C virus; MELD: Model for End-stage Liver Disease.

patient refused reLT (0.7%), reLT not possible due to surgical impediments (0.7%), and unknown (10.8%). The mean follow-up period was of 5796 d (62-9541).

DISCUSSION

Because of the large number of patients with HCV recurrence seen at our transplantation centers, this multicenter and retrospective study is the first non-registry study to have been performed on reLT. Furthermore, it is also the first to have analyzed detailed virological data and antiviral therapies in this population, giving great importance to this factor when selecting patients with HCV recurrence for reLT, especially in the current era of direct-acting antivirals. In this context, the principal prognostic factors associated with the survival in our cohort were: negative HCV viremia before reLT, antiviral therapy after reLT, non-genotype 1, re-listing at MELD below 25, and a reLT donor age < 60 years. The non-retransplanted group was helpful in trying to explain the selection bias for and against an indication for reLT, as the decision to retransplant a patient with HCV recurrence depended on each center's policies.

The most important contribution of our study is the evidence concerning the considerable influence of antiviral therapy before and after reLT. Previous studies had demonstrated the importance of treating HCV before and after LT1[38-47]. During the period of our study, the new drugs that became available were protease inhibitors, and we included only one patient under sofosbuvir, one of the newer direct-acting antivirals. In the modern era of direct-acting antivirals, encouraging results have been achieved using interferon-free HCV regimens in the population of patients with advanced cirrhosis on the waiting list, with high SVR rates and low rates of serious adverse events requiring treatment discontinuation[48,49]. Studies of post-LT treatment with the new DAAs studies have demonstrated SVR rates ranging from 70%-94%[49-53]. Although the numbers in our study were small, we were also able to show that in the 14 patients undergoing reLT with undetectable HCV viremia, the reLT donor age and MELD score did not influence outcome. In the long-term, early antiviral treatment post-LT may become the standard-of-care and reduce the occurrence of advanced graft cirrhosis. However, the subpopulations of individuals presenting with genotype 3, end-stage renal disease, and resistance to DAAs remain a concern.

Several early studies had described high mortality rates in individuals retransplanted for HCV recurrence[25,26], with more recent studies producing survival rates similar to ours[27,54]. Our reLT survival rates of 55%, 47%, and 43% at 3, 5, and 10 years, respectively, were not excessively disappointing given the LT survival rates for HCV cirrhosis alone reported in the literature, which average 75%, 65%, and 52% at 1, 3, and 5 years[55]. A minimum acceptable threshold for graft survival is difficult to define[56]. Previous meetings have suggested a minimum 5-year survival of 50% for reLT[57]. At 5 years, the patients in our cohort achieved a 47% survival rate, but no defined criteria for re-listing were applied. Better patient selection should, therefore, improve survival. Although the survival of the four HIV co-infected patients after reLT was extremely poor (no patient was alive at 3 years), in the context of new DAA, the response rates are promising in the non-transplanted population[58,59]. In a recent multicenter study on reLT in HIV-infected individuals, only four patients experienced an HCV recurrence requiring reLT, even though DAAs were not yet available[60]. Regarding the disappointing survival rates seen in non retransplanted patients compared to those undergoing reLT, many factors may have influenced this result, such as higher rates of SVR in the reLT group and possibly higher rates of comorbidities leading to contraindications for reLT.

In McCashland's study[27], although the MELD score was not predictive of survival, higher MELD scores pre-reLT (> 25) were associated with mortality rates. However, that study was not designed to analyze predictive factors for survival. Our data analyses found that relisting with a MELD > 25 was associated with a poor prognosis but not the MELD score before reLT. In their no-reLT group with decompensated HCV recurrence, survival reached 47% at 3 years, compared to 27% in our study. The percentage of relisted patients in their study was similar to our findings (15% *vs* 12%).

One known factor to improve survival in the setting of HCV-related diseases is donor age[61], and our data confirmed this finding in the context of reLT. Our estimation of survival not only demonstrated the importance of antiviral therapy but also the impact of donor age. This finding triggers another discussion regarding the futility of the procedure in an era of young donor shortages[62]. Although the numbers are small, we also showed that outcomes in patients who underwent reLT with undetectable HCV viremia did not depend on donor age. This issue warrants future evaluation in the context of the availability of new antivirals.

In the registry study by Andres *et al*[29], which proposed the score applied to our patients, several variables were not available from this registry, such as HCV genotype, level of viremia, type of anti-HCV treatment, and biopsy scores, all of which could have markedly affected survival. In our study, patients with an Andres score over 40 had a 5-year survival rate of 40%, compared to an estimated survival of 27% in their cohort. One possible explanation for these discrepant results may have been their inclusion criteria (more than 30 d after reLT for all HCV-positive retransplanted cases), so that they did not solely include patients with HCV recurrence. In addition, our cohort may have included more severely ill patients. Our study was not designed to validate the score, as statistically the number of cases was still too small to offer new thresholds. Other previously published scores had either not been designed specifically for reLT in the event of HCV recurrence[12,32] or were derived from LT1 cases and then extended to reLT and, therefore, did not focus on this population[14].

Our study did have some limitations. Considering that surgical techniques, immunosuppression regimens, antiviral treatment, anesthesiology, and intensive care have changed over the years, the long period we covered could have influenced survival. Notably, the survival rates were similar when our analysis compared reLT before and after 2003. Although our study included data from eight different liver transplantation centers, two of them contributed more than half of the reLT cases (57/108; 53%). As these two centers could be considered more experienced in this technique, an analysis was performed after adjusting for the center effect. No uniform criteria exist for reLT indication in HCV recurrence, and each center adopted its own policies. This analysis also applied to antiviral therapy before and after reLT. Another limitation was that the identification of cases for the non-reLT group was based on histological biopsies post-LT1, but because one center did not perform protocol biopsies, this group may have been under-represented. Furthermore, FCH was defined according to the pathologist's report, and, hence, may not have strictly followed previously accepted definitions of FCH[63-65].

In conclusion, reLT in the context of HCV recurrence requires careful patient selection. From this present study, the first to analyze such detailed virological and treatment data, we can conclude that antiviral therapy both before and after reLT can play an important role when deciding whether to retransplant or not. In an era of new direct-acting antivirals agents, the scenario of retransplantation for HCV recurrence will most likely change dramatically in the future. The findings of our study could nevertheless be useful in the medium term while HCV recurrence is still prevalent, especially in limited-resource settings where direct-acting antivirals are not yet available.

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COMMENTS

Background

Advanced liver disease caused by hepatitis C virus (HCV) is the leading cause of liver transplantation (LT) in Western countries. Histologically documented chronic hepatitis C develops in approximately 70% of patients during the first year after LT. Progression of this disease is particularly aggressive in transplanted patients, with a rapid evolution towards fibrosis when compared to immunocompetent individuals, resulting in graft loss due to recurrent disease. In patients with established cirrhosis and graft failure, retransplantation (reLT) is the only therapeutic option. Because of organ shortages, cost issues, and poor survival, the indications for reLT must be appropriate. In previous studies, HCV-related disease did not indicate a poorer prognosis following reLT, while other studies have identified HCV recurrence as an independent predictive factor for mortality. Few studies, however, have evaluated risk factors for mortality among patients retransplanted for HCV recurrence.

Research frontiers

Retransplantation for HCV recurrence has been a controversial issue because of the possibility of poorer survival compared to other indications. The published literature is scarce on this topic, as there are currently no formal recommendations as to which patients should undergo retransplantation in this context. A multicenter study was performed in order to analyze survival factors in this population.

Innovations and breakthroughs

This is the first published study with a fair number of retransplantations for HCV recurrence to analyze, in terms of virological findings and HCV antiviral therapy, risk factors for improving better prognosis. In view of the new era of direct-acting antivirals and donor scarcity, the results of this study should aid in decision-making in the context of indications for retransplantation.

Applications

In this new era of direct-acting antivirals and donor scarcity, the results of this study should aid with decision-making in the context of indications for retransplantation.

Terminology

The recurrence of hepatitis C is universal among individuals transplanted for HCV-related cirrhosis and with a positive viral load prior to transplantation. Retransplantation may be indicated in those patients who progress to cirrhosis after HCV recurrence on the graft.

Peer-review

This article addresses is a very important topic in the era of chronic organ shortage. The authors have very nicely written a review of 108 patients retransplanted for HCV recurrence in eight European liver transplantation centers and analyzed the factors associated with survival in this population.

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ORIGINAL ARTICLE

Retrospective Study

Video capsule endoscopy in left ventricular assist device recipients with obscure gastrointestinal bleeding

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Abstract

AIM: To assess whether video capsule endoscopy (VCE) affects the outcomes of left ventricular assist devices (LVADs) recipients with gastrointestinal bleeding.

METHODS: This is a retrospective study of LVAD recipients with obscure gastrointestinal bleeding (OGIB) who underwent VCE at a tertiary medical center between 2005 and 2013. All patients were admitted and monitored with telemetry and all VCE and subsequent endoscopic procedures were performed as inpatients. A VCE study was considered positive only when P2 lesions were found and was regarded as negative if P1 or P0 were identified. All patients were followed until heart transplant, death, or the end of the study.

RESULTS: Between 2005 and 2013, 30 patients with LVAD underwent VCE. Completion rate of VCE was 93.3% and there was no capsule retention. No interference of VCE recording or the function of LVAD was found. VCE was positive in 40% of patients (n = 12). The most common finding was active small intestinal bleeding (50%) and small intestinal angiodysplasia (33.3%). There was no difference in the rate of recurrent bleeding between patients with positive and negative VCE study (50.0% *vs* 55.6%,

P = 1.00) during an average of 11.6 ± 9.6 mo follow up. Among patients with positive VCE, the recurrent bleeding rate did not differ whether subsequent endoscopy was performed (50% *vs* 50%, *P* = 1.00).

CONCLUSION: VCE can be safely performed in LVAD recipients with a diagnostic yield of 40%. VCE does not affect recurrent bleeding in LVAD patients regardless of findings.

Key words: Heart-assist devices; Capsule endoscopy; Gastrointestinal hemorrhage; Heart failure; Endoscopy; Digestive system

Core tip: Obscure gastrointestinal bleeding (OGIB) is a common complication for patients receiving left ventricular assist device (LVAD). Although video capsule endoscopy (VCE) is frequently used to investigate OGIB, there is limited data on the safety and usefulness of VCE in LVAD recipients. We found that VCE can be safely performed in LVAD recipients with OGIB and with a 40% diagnostic yield. However, the results of VCE and the subsequent management driven by VCE did not affect the rate of recurrent GIB. Endoscopic intervention thus should be used judiciously, and alternative ways of management should be considered in LVAD patients with OGIB.

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INTRODUCTION

Approximately 50000 patients die from advanced heart failure in the United States each year, with a high mortality rate and life expectancy < 2 years when only medical therapies are utilized[1]. Although heart transplantation is a definitive therapeutic option for advanced heart failure, only 2200 heart transplants are performed annually due to donor shortage, leaving a large proportion of heart failure patients in need of an alternative therapy[2]. In recent years, Left Ventricular Assist Devices (LVADs) are used increasingly in this setting as destination therapy, bridge to transplantation, bridge to recovery, or bridge to decision in patients with advanced heart failure[3]. This approach has increased survival and improved quality of life in advanced heart failure patients[4].

Since the initial introduction of LVAD therapy, it is well documented that LVADs increase the risk of gastrointestinal bleeding (GIB), with as many as 20%-40% of LVAD recipients manifesting GIB[5-7]. The mechanism of GIB in LVAD recipients remains incompletely understood, but it is thought to be contributed to by development of angiodysplasia, acquired von Willebrand disease, persistent right ventricular dysfunction, and mucosal ischemia secondary to low pulse pressure[8,9]. GIB is further exacerbated by the use of anticoagulation in LVAD patients. Although previous reports found that upper GI tract is the most common site of GIB in LVAD recipients[10], obscure gastrointestinal bleeding (OGIB) remains a frustrating condition frequently encountered in this population.

Video capsule endoscopy (VCE) has made a significant impact on the evaluation of patients with OGIB, with a diagnostic yield of approximately 60%-70%[11]. However, it is a relative contraindication to use VCE in the setting of implanted electrical medical devices[12], and there is limited data on both the usage of VCE in LVAD associated OGIB, as well as the safety of VCE in LVAD patients[13-19].

We retrospectively evaluated our experience with VCE in LVAD patients with OGIB. The aims of our study were to determine the safety and diagnostic yield of VCE, and to assess the outcomes based on management driven by VCE in LVAD recipients.

MATERIALS AND METHODS

Patients

All patients undergoing VCE following implantation of LVAD at Washington University Medical Center between January 2005 and September 2013 were eligible for inclusion in this retrospective study. For inclusion, all subjects were required to have obscure GI bleeding, which was defined as hematemesis, melena, hematochezia, or anemia with positive fecal occult blood, without a definitive source identified on upper endoscopy (EGD) and colonoscopy, thereby requiring VCE for further localization of the bleeding source. Exclusion criteria consisted of patients aged less than 18 years, patients who did not have both EGD and colonoscopy performed prior to VCE, incomplete data collection and studies with unintelligible data. This study protocol was approved by the Institutional Review Board at Washington University in St. Louis.

Data collection

Inpatient and outpatient charts were reviewed in the institution's electronic medical records to extract demographic data, indications for LVAD, types of LVAD implanted, follow-up and GIB data. Patients were followed until heart transplant, death or the last point of contact in the electronic medical records. Patients lost to follow up were not included in the final analyses. Episodes of recurrent GIB were identified and recorded. Recurrent GIB was defined as any recurrence of overt GIB or anemia with positive fecal occult blood. Medical records were reviewed to

Table 1 Characteristics and outcomes of left ventricular assist device recipients undergoing video capsule endoscopy *n* (%)

	Total, *n* = 30	Positive study, *n* = 12	Negative study, *n* = 18	*P* value
Age (yr)	60.1 ± 10.2	62.6 ± 8.8	58.4 ± 10.9	0.27
Female	6 (20.0)	4 (33.3)	2 (11.1)	0.18
Charlson comorbidity index	4.7	5	4.6	0.70
History of GIB prior to LVAD	3 (10.0)	2 (16.7)	1 (5.6)	0.55
LVAD implant to GIB (mo)	4.8 ± 6.0	5.5 ± 7.0	4.4 ± 5.3	0.65
Overt GIB	23 (76.7)	8 (66.7)	15 (83.3)	0.39
Antiplatelet agents	26 (86.7)	10 (83.3)	16 (88.9)	1.00
Anticoagulants	28 (93.3)	11 (91.7)	17 (94.4)	1.00
Endoscopies prior to VCE (number)	3.2 ± 1.7	3.5 ± 1.9	3.0 ± 1.7	0.46
Length of stay (d)	13.1 ± 12.5	20.3 ± 17.6	8.3 ± 2.3	0.04
Follow-up (mo)	11.6 ± 9.6	9.2 ± 9.3	13.2 ± 9.7	0.28
Recurrent GIB rate	16 (53.3)	6 (50)	10 (55.6)	1.00
Endoscopies after VCE (number)	1.7 ± 2.2	1.8 ± 1.8	1.7 ± 2.5	0.97
Mortality rate	10 (33.3)	4 (33.3)	6 (33.3)	0.90

GIB: Gastrointestinal bleeding; LVAD: Left ventricular assist device; VCE: Video capsule endoscopy.

determine cause of death, and to determine if death was related to GIB when relevant. The Charlson comorbidity index was calculated based on the review of medical records[20].

Procedures

All VCE studies were performed using PillCam (Given Imaging, Duluth, GA, United States) as inpatients. The risk of capsule retention was assessed by history and radiological imaging studies such as small bowel follow-through or CT enterography per the discretion of GI consult service. Patients were given a half gallon of Golytely (Braintree Laboratories, Braintree, MA, United States) the evening before the procedure and were kept nothing by mouth after midnight. On the day of the procedure, the capsule endoscope was ingested or endoscopically placed in the duodenum if patients had dysphagia or delayed gastric emptying. Patients were monitored by continuous telemetry and evaluated serially by staff. LVADs were monitored continuously by the system controller and interrogated immediately after the VCE *via* the system monitor to evaluate for any changes in function. VCE reports were evaluated for possible LVAD interference and medical records were evaluated for possible LVAD dysfunction related to VCE interference.

Outcomes

The findings on VCE were categorized into 3 types of mucosal abnormalities as previously reported[21]. P0 lesions were those considered to have no bleeding potential such as normal study, submucosal vein, diverticula without bleeding, or nodule without mucosal break. P1 lesions were those having uncertain bleeding potential such as erosions or red spots. P2 lesions were those thought to have high bleeding potential such as ulcers, angiodysplasias, tumors, as well as active bleeding without lesions identified. The diagnostic yield of the study was assessed by the frequency of P2 lesions. Positive VCE studies were defined as VCE findings with P2 lesions. VCE findings reported P0 or P1 lesions were considered as negative VCE studies. If VCE did not reach the cecum at the end of recording, it was considered an incomplete study. Safety endpoints included interference of VCE with LVAD function, interference of LVAD with VCE reports, and other previously described adverse events associated with VCE.

Statistical analysis

For statistical analysis, data is reported as mean ± SD unless otherwise indicated. Fisher's exact test and Student's *t*-test were used for categorical variables and continuous variables, respectively. A *P*-value less than 0.05 was required for statistical significance. Logistic regression was used to examine predictors for VCE outcomes.

RESULTS

Thirty LVAD patients underwent VCE over the 8-year study period. No patient was lost to follow up or excluded in this study. All patients were treated and all procedures were performed as inpatients. The mean age was 60.1 ± 10.2 years, and 20% of patients were female (Table 1). Thoratec HeartMate Ⅱ LVADs were implanted in all of the patients except one patient who had a HeartWare HVAD. The Charlson comorbidity index, the history of GIB prior to LVAD implantation, the interval between LVAD implantation and GIB, and the history of overt GIB did not differ between patients with positive *vs* negative VCE studies. Twenty-three out of the thirty patients (76.7%) presented with overt OGIB: 21 with melena (70%), 2 with hematochezia (6.7%); whereas 7 patients (23.3%) presented with occult OGIB. Most of our patients received antiplatelets (86.7%) or anticoagulants (93.3%) on presentation. On average 3.2 ± 1.7 endoscopic procedures were performed within 4.1 ± 5.0 d prior to VCE, including 37 EGDs, 17 push enteroscopies, 40 colonoscopies,

Table 2 Locations and findings of positive video capsule endoscopy studies *n* (%)

	Patients (*n* = 12)
Locations of positive VCE findings	
Stomach and duodenum	2 (16.7)
Small intestine	9 (75.0)
Colon	1 (8.3)
Findings of positive VCE studies	
Small intestinal bleeding with no source or lesion identified (2 in the duodenum, 4 in the jejunum)	6 (50.0)
Angiodysplasia (1 in the duodenum, 3 in the small bowel)	4 (33.3)
Colonic bleeding with no source or lesion identified	1 (8.3)
Gastric ulcer	1 (8.3)

VCE: Video capsule endoscopy.

and 2 sigmoidoscopies. VCE was performed 6.2 ± 2.6 d after the presentation of GIB. VCE was placed endoscopically in 2 patients (6.7%) because one patient had a history of pyloric stenosis, and the other patient failed the swallow study. The mean small bowel transit time of VCE was 3.2 ± 1.1 h. VCE did not reach the cecum in 2 patients (6.7%) over the 8 h recording period, but there was no capsule retention. There was no electromagnetic interference of either VCE or LVAD identified in any patients.

Patients with positive VCE study stayed in the hospital longer than patients with negative VCE study (20.3 d *vs* 8.3 d, $P = 0.04$). Over the average 11.6 mo follow-up period, there was no statistically significant difference in the recurrent bleeding rate (50% *vs* 55.6%, $P = 1.00$), the number of endoscopies performed after VCE (1.8 ± 1.8 *vs* 1.7 ± 2.5, $P = 0.97$), or mortality rate (33.3% *vs* 33.3%, $P = 0.90$) between patients with positive and negative VCE. The total recurrent bleeding rate in this population was 53.3% ($n = 16$) and the presentation included melena ($n = 12$), hematochezia ($n = 3$) and anemia with positive fecal occult blood ($n = 1$). All 16 patients with recurrent bleeding were hospitalized and underwent transfusion and endoscopic procedures for managing recurrent GIB. The overall mortality rate in this study was 33.3% ($n = 10$): 7 patients died from underlying heart failure, 2 patients died from septic shock, one patient died from subdural hematoma, and none of the patients died from GIB. Four LVAD recipients underwent heart transplantation on average 4.3 mo after VCE and did not develop recurrent GIB afterwards. Before heart transplantation, VCE studies were positive in 2 patients (1 duodenal angiodysplasia and 1 jejunal angiodysplasia) and negative in 2 others.

The diagnostic yield of VCE to detect P2 lesions in this study was 40%. Table 2 demonstrates the locations and the findings of positive VCE studies. Small intestine was the most common site of positive VCE findings (75%). The predominant positive VCE findings in our study were small intestinal bleeding with no source or lesion identified (50%) and small intestinal angiodysplasias (33.3%). Eighteen VCE studies (60%) were negative, including 13 P0 lesions (12 normal, 1 small nodule) and 5 P1 lesions (red spots). The only patient who had a HeartWare HVAD implanted had a normal VCE study. Despite prior negative upper endoscopies performed 2 and 24 d prior to VCE, two lesions were found within reach of EGD by VCE: one gastric ulcer and one duodenal angiodysplasia (neither VCE was placed endoscopically). One VCE found active bleeding in the colon without the cause of bleeding identified. Angiodysplasia were found in 4 patients: 1 in the duodenum and 3 in the small intestine. In 2 patients where VCE failed to reach the cecum at the end of recording, VCE still detected the cause of GIB: one with gastric ulcer and one with small intestine angiodysplasia. Using logistic regression, we found that higher INR on presentation was associated with a higher probability of positive findings in VCE (OR = 3.62, 95%CI: 1.03-12.7, $P = 0.04$), adjusted for age, gender, and hemoglobin level.

Positive VCE studies led to further endoscopic evaluations in 6 patients out of 12 (50%): 6 push endoscopies and 3 single balloon enteroscopies. The other 6 patients with positive VCE did not have further endoscopies because they had no further bleeding and their hemoglobin had stabilized. During follow-up the overall recurrent bleeding rate in patients with positive VCE was 50% (6 out of 12). In addition, there was no difference in the recurrent bleeding rate whether subsequent endoscopic procedures were performed following positive VCE (3 out of 6 or 50% in each group, $P = 1.00$). Furthermore, after VCE, medications were adjusted in 7 out of 12 patients with positive VCE, and 8 out of 18 patients with negative VCE. These changes included discontinuation or decrease in the dose of aspirin and initiation of proton pump inhibitors. The change of medical management did not affect the rate of recurrent bleeding regardless of whether patients had a positive VCE (40% *vs* 57.1%, $P = 1.00$), or negative VCE (60% *vs* 50%, $P = 1.00$). The presentations of recurrent GIB were melena ($n = 4$), hematochezia ($n = 1$) and anemia with positive fecal occult blood ($n = 1$). The clinical course and management of LVAD recipients with positive VCE studies are detailed in Table 3.

DISCUSSION

In this retrospective study spanning 8 years, we demonstrated the safety of VCE and a 40% diagnostic yield of P2 lesions in LVAD recipients with OGIB. We found that the results of VCE were not associated with the rate of recurrent GIB, the number of endoscopic procedures performed, or mortality rate. In addition, the findings of VCE and the subsequent management did not affect the rate of recurrent GIB in LVAD patients.

This study identified 30 LVAD recipients undergoing

Table 3 Clinical course and management of left ventricular assist device recipients with positive video capsule endoscopy studies

No.	Age (yr) and Sex	Presentation	Endoscopic findings and interval prior to VCE (d)	VCE findings	Endoscopic findings and interval after VCE (d)	Recurrent bleeding and interval after VCE (mo)	Management for recurrent bleeding
1	62 M	Anemia	Rectal polyp (2)	Gastric ulcer	N/A	Melena (21)	EGD: GU with visible vessel s/p hemoclip
2	57 M	Anemia	Gastritis, colonic polyp (24)	Duodenal angiodysplasia	PE: Gastritis (2)	No	N/A
3	73 M	Melena	Gastric and jejunal angiodysplasia (4)	Small bowel angiodysplasia	N/A	Melena (1.7)	PE: Bleeding jejunal angiodysplasia s/p APC
4	53 F	Melena	Blood in the terminal ileum (0)	Small bowel angiodysplasia	PE: Bleeding jejunal angiodysplasia s/p APC + hemoclip (2)	No	N/A
5	61 M	Anemia	Colonic diverticulosis, hemorrhoids (3)	Small bowel angiodysplasia	N/A	No	N/A
6	53 M	Anemia	Duodenitis (4)	Stomach and small bowel bleeding	PE: Clean base GU (2)	Melena (0.5)	PE: Gastritis and fresh blood in duodenum without lesions identified
7	70 F	Melena	Gastric angiodysplasia s/p APC (3)	Small bowel bleeding	N/A	Melena (11.4)	PE: DLBCL of stomach
8	61 F	Hematochezia	Sigmoid angiodysplasia s/p APC (2)	Small bowel bleeding	PE: Normal; SBE: Bleeding jejunal angiodysplasia s/p APC+ hemoclip (2)	Hematochezia (11.2)	PE: Gastritis; C-scope: Bleeding sigmoid angiodysplasia s/p APC + hemoclip
9	52 M	Melena	Colonic diverticulosis, hemorrhoids (1)	Small bowel bleeding	N/A	No	N/A
10	59 M	Melena	Duodenal angiodysplasia s/p APC and hemoclip (5)	Small bowel bleeding	PE: Bleeding jejunal angiodysplasia s/p heater probe + APC; SBE: Nonbleeding jejunal angiodysplasia s/p APC (6)	Anemia requiring transfusion (0.7)	C-scope: Bleeding Cecal angiodysplasia s/p hemoclip
11	75 M	Melena	Colonic angiodysplasia s/p hemoclip (6)	Small bowel bleeding	PE: Gastric Dieulafoy's lesion s/p hemoclip; SBE: Bleeding jejunal angiodysplasia s/p APC + hemoclip (2)	No	N/A
12	76 F	Melena	Normal (4)	Colonic bleeding	N/A	No	N/A

APC: Argon plasma coagulation; C-scope: Colonoscopy; DLBCL: Diffuse large B-cell lymphoma; EGD: Esophagogastroduodenoscopy; F: Female; GU: Gastric ulcer; M: Male; N/A: Not applicable; PE: Push enteroscopy; SBE: Single balloon enteroscopy; VCE: Video capsule endoscopy.

VCE for OGIB. To our knowledge, this is the largest available series of VCE in LVAD patients in the literature[6,13-19,22] (Table 4). Our results show that VCE is safe to perform in LVAD recipients, without interference between VCE and LVAD and without capsule retention. One prior study found 2 cases of LVAD possibly interfering with capsule images, and suggested that the leads of VCE be placed away from LVAD[23]. Based on the results of this and other previous studies, the interference between VCE and LVAD is uncommon[14,16,23]. Consistent with our experience, a recent review article reported that VCE is unlikely to impair the function of cardiac pacemakers, implantable cardioverter defibrillators, and LVAD, although the authors cautioned that wireless telemetry may interfere with VCE recordings[24].

The diagnostic yield of VCE in LVAD patients in two previous reports was 31% (n = 13) and 80% (n = 5) (Table 4)[6,22]. With a larger sample size of 30, the diagnostic yield in our study is 40%. Since only P2 lesions were considered positive, our diagnostic yield reflects true clinically relevant findings. It is known that the diagnostic yield of VCE is higher when it is performed closer to the presenting GIB event[25,26], or in patients with overt GIB than with occult GIB[27,28]. It is also known that the diagnostic yield of VCE is comparable to double balloon enteroscopy (DBE) according to a meta-analysis[29]. A recent study of DBE in LVAD recipients who presented with overt OGIB found that the diagnostic yield of DBE was 69% when DBE was performed within 24 h of initial presentation[30]. We suspect that VCE would have a similar diagnostic yield if it is performed within 24 h of overt OGIB. In our study, VCE was performed on average 6.2 d after admission- after coagulopathy was corrected and after other endoscopic procedures failed to identify the cause of OGIB. Using logistic regression, we found that higher INR on presentation was associated with a higher probability of positive VCE. If OGIB is highly suspected in an LVAD patient with a supra-therapeutic INR on presentation, expediting VCE, possibly before coagulopathy is corrected and

Table 4 Previous studies of video capsule endoscopy in left ventricular assist device recipients

Study and the year published	No. of VCE	Diagnostic yield (%)	Findings	Remarks
Girelli *et al*[18], 2006	1	N/A	No bleeding identified	No follow up reported
Garatti *et al*[15], 2006	1	N/A	No bleeding identified	No recurrent GIB; received heart transplantation
Seow *et al*[17], 2006	1	N/A	Duodenal and jejunal angiodysplasia	Push enteroscopy + Octreotide + Sucralfate; no follow up reported
Fenkel *et al*[16], 2007	1	N/A	Small bowel angiodysplasia	No intervention; no follow up reported
Daas *et al*[14], 2008	1	N/A	Mid-small bowel active bleeding	Intraoperative enteroscopy; no follow up reported
Bechtel *et al*[19], 2010	1	N/A	Bleeding in cecum	Colonoscopy; no follow up reported
Elmunzer *et al*[6], 2011	13	4 (30.8)	3 jejunal angiodysplasias, 1 duodenal Dieulafoy's lesion	1 recurrent bleeding from the same lesion
Meyer *et al*[22], 2012	5	4 (80.0)	2 jejunal angiodysplasia, 1 cecal ulcer, 1 jejunal mass	1 colonoscopy; 2 SBE; 1 angiography; no follow up reported
Tarzia *et al*[13], 2013	1	N/A	Small bowel angiodysplasia and small bowel Dieulafoy's lesion	DBE; no follow up reported

DBE: Double balloon enteroscopy; GIB: Gastrointestinal bleeding; N/A: Not applicable; SBE: Single balloon enteroscopy; VCE: Video capsule endoscopy.

other endoscopies performed, may improve the diagnostic yield of VCE in this population.

In a general population with OGIB, > 80% of the bleeding sites are in the small bowel[11]. The most common location of positive VCE finding in our LVAD patients with OGIB is also small bowel (75%). The most common finding of positive VCE in our study was small bowel bleeding without the cause of bleeding identified, whereas small bowel angiodysplasia was the second most common finding. The source of bleeding can be difficult to identify by VCE in the setting of active bleeding since blood can obscure visualization and VCE cannot clear the visual field with water irrigation. Nevertheless, we surmise that angiodysplasia is the most likely cause of GIB in those with small bowel bleeding without lesions identified given that it has been shown to be the most common cause of GIB in LVAD patients[6,7,22]. Indeed, 3 patients with small bowel bleeding without lesion identified on VCE were later found to have bleeding small bowel angiodysplasias on subsequent enteroscopies. Of note, in our study 25% of positive VCE findings were within reach of EGD or colonoscopy, underscoring the elusive nature of these lesions and the importance of repeat examinations if necessary.

Our study is the first to report recurrent GIB rate in LVAD recipients after VCE. Although LVAD is thought to predispose patients to GIB through various mechanisms, the recurrent GIB rate (53%) in our LVAD population is similar to OGIB in non-LVAD patients[31]. Furthermore, there was no difference in the rate of recurrent GIB, the number of endoscopies performed after VCE, and the mortality rate, whether VCE studies were positive or negative. Even among patients with a positive VCE, further endoscopic intervention did not affect recurrent bleeding- the recurrent bleeding rate was 50% regardless of endoscopic intervention (Table 3). On the other hand, the negative VCE study in our cohort was not associated with lower recurrent bleeding rate. It is possible that OGIB may merely be a reflection of the underlying condition or hemodynamics of LVAD patients, and endoscopic procedures do not necessarily impact their natural courses. Endoscopic evaluation or therapy in this population therefore should be used judiciously, especially given that medical therapy with thalidomide or Octreotide has recently been reported to be effective in LVAD recipients with OGIB[32,33]. Further study is required to identify the characteristics of patients who may benefit from endoscopic intervention *vs* supportive care.

Of the 12 patients with positive VCE studies, only 6 patients had subsequent endoscopic procedures because the other 6 patients had no further GI bleeding and their hemoglobin had stabilized, again indicating a dissociation of VCE findings with patients' clinical courses. Given that only 50% of patients with positive VCE had persistent GIB requiring further endoscopic intervention, one possible approach to treat LVAD recipients with OGIB based on our finding is to defer VCE and endoscopies until persistent GIB is observed. The current health care environment creates tremendous pressure on hospitals to shorten patients' stay and expedite diagnostic procedures and treatment. For certain patients in this population, however, it may be more cost-effective to observe and provide supportive medical care without endoscopies. Future studies are needed to test this hypothesis and to determine which subset of LVAD recipients with OGIB would benefit from VCE and subsequent endoscopies.

None of LVAD recipients in this study died from GIB, consistent with a prior report[34]. All 4 patients who received heart transplantation in our study did not have recurrent bleeding episodes during the study period, which is similar to the result in a recent systematic review that reported 12 patients without recurrent GIB after heart transplantation[7]. All together, the data indicates that the changes of physiology and hemodynamics associated with LVAD are the causes of GIB, and this process and GIB can

be reversed when LVAD is removed. Consistent with this concept, a previous study found that all LVAD recipients had reduced high molecular weight von Willebrand factor multimers which were normalized after patients received heart transplants[35].

Our study has limitations. Given that this is a retrospective study from a single tertiary medical center, it has weaknesses similar to other retrospective studies and should be carefully interpreted or generalized. In addition, although this study provides the largest series of VCE in LVAD recipients with OGIB, the sample size of 30 is still limited. We might underestimate the true rate of recurrent bleeding because the follow-up duration may not be long enough. Lastly, in our study VCE were interpreted by several gastroenterologists and the management of patients was not standardized. These variations may affect the results of our study.

In conclusion, our study shows that VCE can be safely performed in LVAD recipients with OGIB and with a 40% diagnostic yield. However, the results of VCE and the subsequent management driven by VCE did not affect the rate of recurrent GIB. Endoscopic intervention thus should be used judiciously in this patient population. An observation-and-supportive care approach could be an alternative way to treat LVAD patients with OGIB. Future studies should answer the questions of what subset of LVAD recipients with OGIB would benefits from endoscopic therapy, and what the most cost-effective way is to take care of this challenging group of patients.

COMMENTS

Background

Gastrointestinal bleeding (GIB) is one of the most common complications in Left Ventricular Assist Devices (LVADs) recipients. The mechanism of GIB in this setting is still unclear, but it is thought to be the results of development of angiodysplasia, acquired von Willebrand disease, persistent right ventricular dysfunction, and mucosal ischemia secondary to low pulse pressure. Moreover, managements of GIB in patients with LVAD are challenging. It is quite difficult to determine the source of GIB and it is not uncommon to not be able to identify the site of bleeding despite extensive workup. Video capsule endoscopy (VCE) has been used extensively for evaluating patients with obscure GIB (OGIB). In this study, we determined the safety and diagnostic yield of VCE, and assessed the outcomes of GIB based on management driven by VCE in LVAD recipients.

Research frontiers

There is limited data on the usage of VCE in LVAD patients. The results of this study provide evidence on the utility and safety of VCE in LVAD patients.

Innovations and breakthroughs

This study showed that VCE can be safely performed in LVAD recipients and the diagnostic yield of VCE was 40%. However, the results of VCE and the subsequent management driven by VCE did not affect the rate of recurrent GIB.

Applications

Although VCE is relatively safe to perform in LVAD recipients, VCE does not necessarily change the course of OGIB in LVAD recipients. Thus, endoscopic intervention should be used carefully in these patients. An observation-and-supportive care approach could be an alternative way to treat LVAD patients with OGIB.

Terminology

LVAD, an implantable mechanical device that helps a heart pumps blood throughout the body. OGIB, gastrointestinal bleeding that is unable to identify the cause after upper endoscopy and colonoscopy are performed. VCE, a small wireless camera that is ingested to examine parts of GI tract.

Peer-review

This is an interesting study for patients with obscure GI bleeding and LVAD receiving VCE.

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ORIGINAL ARTICLE

Retrospective Study

Methylation of *DAPK* and *THBS1* genes in esophageal gastric-type columnar metaplasia

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Abstract

AIM: To explore methylation of *DAPK*, *THBS1*, *CDH-1*, and *p14* genes, and *Helicobacter pylori* (*H. pylori*) status in individuals harboring esophageal columnar metaplasia.

METHODS: Distal esophageal mucosal samples obtained by endoscopy and histologically diagnosed as gastric-type (non-specialized) columnar metaplasia, were studied thoroughly. DNA was extracted from paraffin blocks, and methylation status of death-associated protein kinase (*DAPK*), thrombospondin-1 (*THBS1*), cadherin-1 (*CDH1*), and *p14* genes, was examined using a methyl-sensitive polymerase chain reaction (MS-PCR) and sodium bisulfite modification protocol. *H. pylori cagA* status was determined by PCR.

RESULTS: In total, 68 subjects (33 females and 35 males), with a mean age of 52 years, were included. *H. pylori cagA* positive was present in the esophageal gastric-type metaplastic mucosa of 18 individuals. *DAPK*, *THSB1*, *CDH1*, and *p14* gene promoters were methylated by MS-PCR in 40 (58.8%), 33 (48.5%), 46

(67.6%), and 23 (33.8%) cases of the 68 esophageal samples. *H. pylori* status was associated with methylation of *DAPK* (P = 0.003) and *THBS1* (P = 0.019).

CONCLUSION: DNA methylation occurs in cases of gastric-type (non-specialized) columnar metaplasia of the esophagus, and this modification is associated with *H. pylori cagA* positive infection.

Key words: DNA methylation; Esophageal columnar metaplasia; Thrombospondin-1; Death-associated protein kinase; *Helicobacter pylori*; *cagA*

Core tip: Columnar metaplasia of the esophagus, whether specialized or not, is a hallmark of gastroesophageal reflux disease. Current information suggests that intestinal metaplasia in the esophagus arises from gastric-type metaplasia. In this study, we have demonstrated that *Helicobacter pylori* (*H. pylori*) *cagA*$^+$ can colonize esophageal gastric-type metaplastic mucosa, and that DNA methylation of tumor suppressor genes could be related to *H. pylori cagA*$^+$ infection, which in turn, may predispose to precancerous lesions, including intestinal metaplasia.

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INTRODUCTION

Columnar metaplasia of the esophagus is defined as the replacement of the normal settled squamous epithelium in the distal portion of the esophagus with columnar epithelium, either with goblet cells [specialized columnar metaplasia, *i.e*., complete or incomplete intestinal metaplasia, Barrett esophagus (BE)], or without goblet cells (non-specialized columnar metaplasia or gastric-type metaplasia; *i.e*., oxyntocardiac and cardiac mucosa)[1]. The importance of such a distinction is found in the higher risk of developing adenocarcinoma of the distal esophagus in patients harboring specialized columnar metaplasia; the incidence of adenocarcinoma originating in BE has been reported as approximately 0.5% per year, with higher risk when long-segment BE is present[2]. However, it is now recognized that the presence of specialized columnar metaplastic cells (goblet cells) is not an essential requirement for the development of such adenocarcinomas[3]. Columnar metaplasia of the esophagus, whether specialized or not, is a hallmark of gastroesophageal reflux disease (GERD), and current information suggests that intestinal metaplasia in the esophagus arises from gastric-type, oxyntocardiac and/or cardiac (non-specialized) metaplasia[4]. Histologically, the non-specialized metaplastic mucosa of the esophagus may display any of the changes commonly observed in settled gastric mucosa, including the changes observed during *Helicobacter pylori* (*H. pylori*) infection[5].

Neoplastic transformation and progression in BE are related with genetic and epigenetic events that ultimately favor abnormal expression of the genes responsible for the intrinsic control mechanisms regulating cellular proliferation and/or apoptosis. DNA methylation is involved in the epigenetic regulation of gene expression (primarily through gene repression) when it occurs predominantly in regions containing CpG islands, which are often concentrated in the promoter regions of genes. In the case of esophageal adenocarcinoma, abnormal methylation patterns are not only detected in neoplastic tissue but also in premalignant Barrett mucosa. These results suggest that hypermethylation of DNA is an early epigenetic event in the multistep process of esophageal carcinogenesis[6].

Recent reports suggested that *H. pylori* is an initiator of the inflammatory microenvironment which might promote carcinogenesis and progression of gastric cancer[7]; in gastric diseases, chronic inflammation and alterations in DNA and histone methylation, especially at promoter regions, are frequently associated with *H. pylori* infection[8,9]. Such epigenetic alteration could be promoted in part by activation of NF-κB and PI3K/AKT-Sp1-RBP2-Cyclin D1 pathways triggered by *H. pylori* Cytotoxin-associated gene product [cytotoxin-associated gene A (*cagA*)] positive[7,9,10]. In addition to aging, increased methylation of several genes has also been described in chronic gastritis and premalignant stages of gastric carcinoma, irrespective of *H. pylori* status[11]. Moreover, *H. pylori* infection induces an overexpression of DNA methyltransferases (DNMTs), which has been associated with CpG island methylation of multiple gene promoters involved in cell growth, differentiation and tumor suppression, like *Ndrg2*, *p14*, *DAPK* and cadherin-1 (*CDH1*), as in chronic gastritis as in gastric cancer[7,12-14].

Consequently, the aim of this retrospective and descriptive study, was to explore the relationship between methylation of the death-associated protein kinase (*DAPK*), thrombospondin-1 (*THBS1*), *CDH1*, and *p14* genes, and the presence of *H. pylori cagA* positive, in the non-specialized, gastric-type columnar metaplastic mucosa of the distal esophagus, in a group of Mexican patients.

Table 1 Sequences of specific primers used for determining gene methylation status

Primer name		Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Product size (bp)	AT (℃)	No. of cycles
DAPK	M	GGATAGTCGGATCGAGTTAACGTC	CCCTCCCAAACGCCGA	98	60	35
	U	GGAGGATAGTTGGATTGAGTTAATGTT	CAAATCCCTCCCAAACACCAA	98	60	35
p14	M	GTGTTAAAGGGCGGCGTAGC	AAAACCCTCACTCGCGACGA	122	60	40
	U	TTTTTGGTGTTAAAGGGTGGTGTAGT	CACAAAAACCCTCACTCACAACAA	132	60	40
CDH1	M	TTAGGTTAGAGGGTTATCGCGT	TAACTAAAAATTCACCTACCGAC	115	53	35
	U	TAATTTTAGGTTAGAGGGTTATTGT	CACAACCAATCAACAACACA	97	57	35
THBS1	M	TGCGAGCGTTTTTTTAAATGC	TAAACTCGCAAACCAACTCG	74	62	40
	U	GTTTGGTTGTTGTTTATTGGTTG	CCTAAACTCACAAACCAACTCA	115	62	40

M: Methylated sequence; U: Unmethylated sequence; AT: Annealing temperature.

MATERIALS AND METHODS

Patients

This is a retrospective and descriptive study of consecutive cases with the histopathological diagnosis of non-specialized (gastric-type), but not specialized (complete or incomplete intestinal metaplasia), columnar metaplasia of the distal esophagus. Biopsies of the distal esophagus, as well as gastric biopsies, were retrieved from the files of the Department of Pathology at the Instituto Nacional de Cancerología (INCan) in Mexico City during the period between January 2003 and December 2008. Inclusion criteria were females and males, aged > 18 years. All biopsies were obtained by means of the panendoscopy procedure at the Endoscopy Service outpatient clinic, in patients with upper gastrointestinal complaints and with endoscopic suspicion of columnar metaplasia. Relevant demographic and clinical data for each patient were retrospectively retrieved from their clinical records. Non-specialized columnar metaplasia of the esophagus was operationally defined as the presence of cardiac and/or oxyntocardiac gastric mucosa lacking goblet cells and intermingled with recognizable islets of non-keratinized squamous epithelium and/or immersed duct structures lined by multilayered epithelium. Slides stained with hematoxylin and eosin (HE) from each case were thoroughly reviewed, and histological criteria for gastritis (mononuclear cells and neutrophils) and *H. pylori* density were applied to grade the samples according to the Visual analog scales (VAS) proposed by the Updated Sydney System[15]. Giemsa staining was also performed to confirm the presence of *H. pylori*. Finally, cases with sufficient tissue were selected for morphological and molecular analysis. DNA was extracted from paraffin-embedded tissue samples.

DNA extraction and bisulfite modification

DNA was extracted from histological sections of 20 μm in thickness by phenol/chloroform/isoamyl alcohol. One μg of genomic DNA extracted from samples was subjected to sodium bisulfite modification using a Zymo Kit (EZ DNA Methylation™ Kit; Zymo Research Co., United States) following manufacturer's instructions for small samples. As a control, we employed human lymphocyte DNA, known to be unmethylated in the promoter regions of our genes-of-interest.

Methylation-specific polymerase chain reaction

Bisulfite-modified DNA was amplified with primers specific for either methylated or unmethylated sequences[11] (Table 1). PCR products were subjected to electrophoresis on 3% agarose gels and were then visualized under ultraviolet (UV) illumination using ethidium bromide.

H. pylori detection by PCR

The presence of *H. pylori* was corroborated by PCR using the following primers from the conserved region of the *cagA* gene: forward, 5'-TT CAT GGG CGT GTT TGA TG-3', and reverse, 5'-AGC GAC TCC CTC AAC ATC TAA-3'. The fragments were amplified with 30 cycles utilizing a 55 ℃ annealing temperature.

Statistical analysis

Statistical review of the study was performed by a biomedical statistician. The association of tested variables with the presence of *H. pylori* infection was assessed using the χ^2 test. OR with their corresponding 95%CIs were calculated as a measure of association using logistic regression analysis. Two-sided statistics were used in all cases, and a probability (*P*) value of 0.05 was considered as significant. SPSS ver. 19 software (2010; IBM Corp., Armonk, NY, United States) was used for computations.

RESULTS

A total of 68 subjects (33 females and 35 males) with a mean age of 52 years (range, 25-88 years) fulfilled the histological criteria and were thus eligible for the study. In 35 subjects, gastric mucosa samples were insufficient for the molecular study, and in the remaining 33 (48.5%) subjects, the gastric samples were processed for final analysis. Endoscopic findings at the distal esophagus included variable degrees of mucosal erosion, salmon-colored mucosal tongues and irregular Z line. Histologically, all cases showed chronic inflammation. In the esophageal samples, the

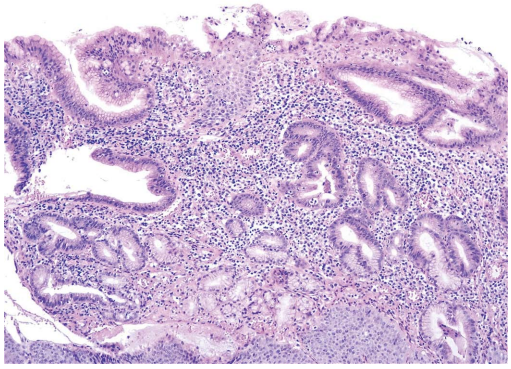

Figure 1 Islets of non-keratinized squamous epithelium can be observed intermingling with cardiac-type gastric mucosa. Superficial and glandular gastric epithelial linings demonstrate infiltration by polymorphonuclear leukocytes (Hematoxylin-eosin staining; original magnification × 10).

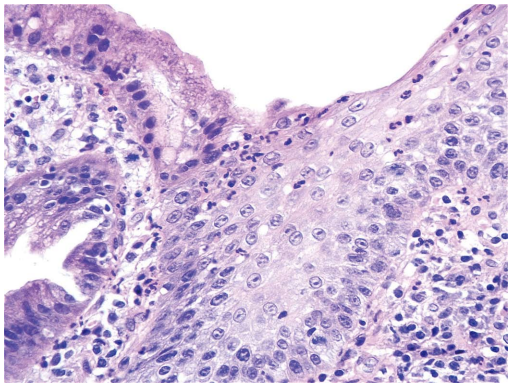

Figure 2 Histological section of gastric-type columnar mucosa of the esophagus. Moderate mononuclear infiltrate in the lamina propria and polymorphonuclear leukocytes infiltrating the mucosa layer can be observed (Hematoxylin-eosin staining stain; original magnification × 40).

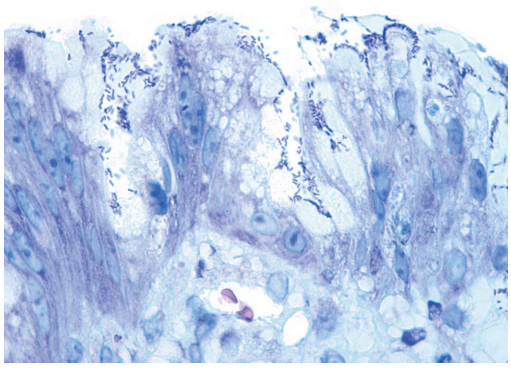

Figure 3 Numerous rod-shape bacilli can be seen attached to the gastric-type metaplastic columnar epithelium (Giemsa staining; original magnification × 100).

intensity of the mononuclear infiltrate was moderate in 43 (63.2%) cases, mild in 18 (26.5%), and marked in 7 (10.3%). Forty (58.8%) of the cases displayed polymorphonuclear leukocyte activity in the gastric-type metaplastic mucosa, which was graded as mild in 33 (82.5%) cases, moderate in 5 (12.5%), and marked in 2 (5%) (Figures 1 and 2). In 22 (32.3%) cases, *H. pylori* microorganisms were identified at the luminal surface of the gastric-type metaplastic mucosa. *H. pylori* density was graded as mild in 17 (77.3%) cases, moderate in 3 (13.6%), and marked in 2 (9.1%) (Figure 3). Regarding the 33 gastric biopsies, mononuclear infiltrate intensity was moderate in 16 (48.5%) cases, mild in 14 (42.4%), and marked in 3 (9.1%). Polymorphonuclear leukocyte activity was present in 17 (51.5%) cases and graded as moderate in 8 (47%), mild in 7 (41.2%), and marked in two (11.8%). *H. pylori* microorganisms were found in 17 (51.5%) cases and graded according to density, as moderate in 10 (58.8%), mild in 5 (29.4%), and marked in two (11.8%). In addition, four (12.1%) and two (6%) of the 33 cases displayed mild and moderate complete intestinal metaplasia, respectively. In one (3%) case, there was also mild atrophy of the gastric mucosa.

Table 2 Association of *Helicobacter pylori cagA*$^+$ infection as detected by PCR, with DNA methylation of the promoter regions of target genes in the esophageal biopsies

		Helicobacter pylori infection by PCR		*P* value
		Negative	Positive	
DAPK	Unmethylated	26	2	0.003[1]
	Methylated	24	16	
THBS1	Unmethylated	30	5	0.019[1]
	Methylated	20	13	
CDH1	Unmethylated	19	3	0.097
	Methylated	31	15	
p14	Unmethylated	36	9	0.091
	Methylated	14	9	

[1]*Helicobacter pylori cagA*$^+$ status was significantly associated with methylation.

CagA$^+$ *H. pylori* was detected in the esophageal non-specialized metaplastic mucosa and in the gastric mucosa of 18 (26.5%) of 68 individuals, and in 10 (30.3%) of 33, respectively, by means of Polymerase chain reaction (PCR) (Figure 4A). *DAPK*, *THSB1*, *CDH1*, and *p14* gene promoters were methylated by MS-PCR in 40 (58.8%), 33 (48.5%), 46 (67.6%), and 23 (33.8%) cases of the 68 esophageal samples and in 10 (30.3%), 14 (43.8%), 26 (78.8%), and 10 (31.3%) of the gastric biopsies, respectively. In the remaining cases, these genes were not methylated (Figure 4B). *H. pylori cagA*$^+$ status was significantly associated with methylation of *DAPK* (P = 0.003) and *THBS1* (P = 0.019) in the 68 esophageal samples (Table 2), and bivariate analysis confirmed the significance of this association (Table 3). In the comparative analysis between the 33 gastric and 33 esophageal paired samples, the trend for the association between *H. pylori cagA*$^+$ and methylation of *THBS1* and *DAPK* genes was maintained (Table 4). Methylation of the *CDH1* and *p14* gene promoters did not exhibit statistically significant differences between *H. pylori cagA*$^+$ and *cagA*$^-$ cases, in both the esophageal and

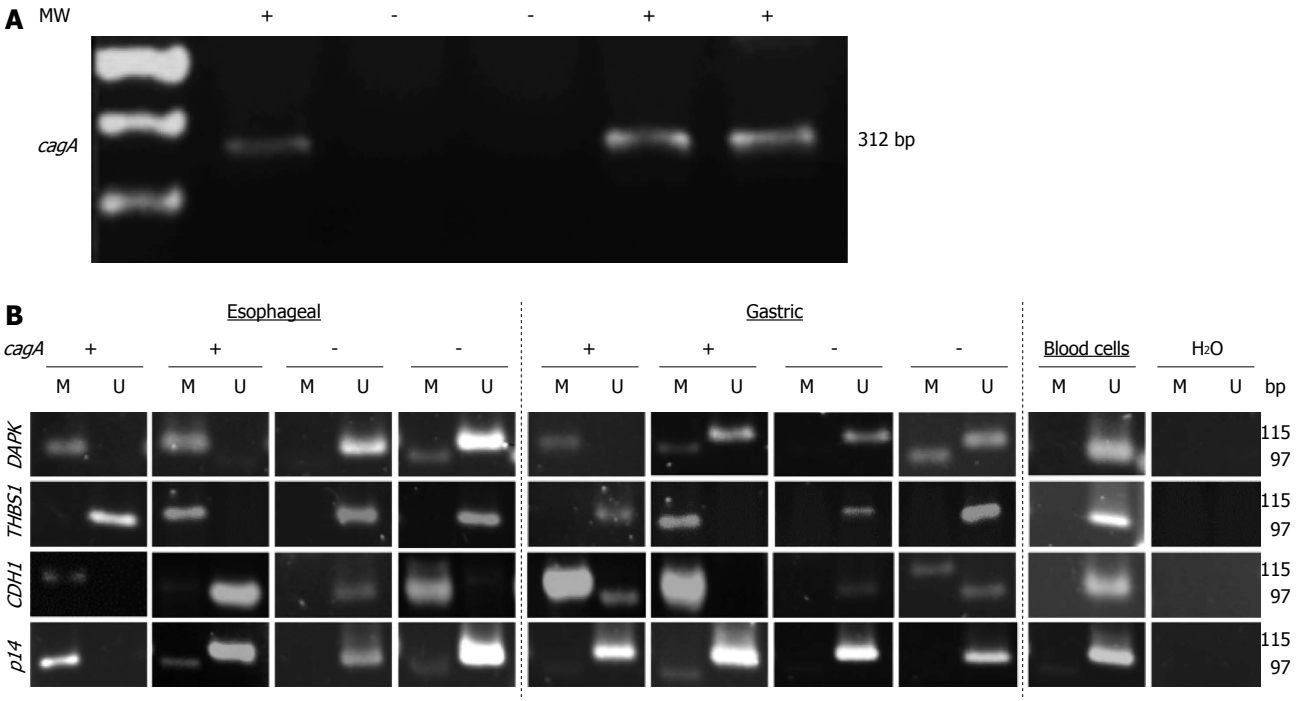

Figure 4 *cagA* detection and DNA promoter methylation status of *DAPK*, *THBS1*, *CDH1* and *p14* genes in esophageal and gastric mucosa tissue samples. A: *Helicobacter pylori cagA*$^+$ detection by polymerase chain reaction (PCR) amplification in tissue samples, (+) positive and (-) negative; B: Analysis of methylation status of *DAPK*, *THBS1*, *CDH1*, and *p14* genes promoters in esophageal mucosa and gastric biopsies tissue samples detected by methyl-sensitive PCR. Blood cells were used as control of unmethylated promoter regions. M represents methylated and U represent unmethylated; the status of *Helicobacter pylori cagA* is indicated as (+) positive and (-) negative.

Table 3 Bivariate analysis of the association between molecular markers and the presence of *Helicobacter pylori cagA*$^+$ infection in esophageal biopsies

	OR	95%CI	*P* value
DAPK	8.67	1.80-41.7	0.007[1]
THBS1	3.90	1.20-12.6	0.023[1]
CDH1	3.06	0.78-11.9	0.110
p14	0.39	0.13-1.18	0.096

[1]Statistically significant.

Table 4 Association of *Helicobacter pylori cagA*$^+$ infection as detected by PCR with DNA methylation of the promoter regions of target genes in 33 esophageal and gastric biopsies

		Helicobacter pylori infection by PCR		*P* value
		Negative	Positive	
Esophageal biopsies				
DAPK	Unmethylated	14	0	0.004[1]
	Methylated	10	9	
THBS1	Unmethylated	15	2	0.057[1]
	Methylated	9	7	
CDH1	Unmethylated	11	1	0.107
	Methylated	13	8	
p14	Unmethylated	18	5	0.400
	Methylated	6	4	
Gastric biopsies				
DAPK	Unmethylated	17	6	0.444
	Methylated	6	4	
THBS1	Unmethylated	15	3	0.062
	Methylated	7	7	
CDH1	Unmethylated	6	1	0.397
	Methylated	17	9	
p14	Unmethylated	14	8	0.440
	Methylated	8	2	

[1]Statistically significant.

the gastric biopsies. Among the esophageal biopsies, there were no significant differences regarding the age (P = 0.39) or gender (P = 0.34) of the subjects and *H. pylori* status by means of PCR; histopathological variables according to the Updated Sydney System for the classification and grading of gastritis were significantly associated with *H. pylori cagA*$^+$ status (Table 5). On the other hand, *H. pylori* density, mononuclear cells, and neutrophils, as histologically graded according to the Updated Sidney System for the classification of gastritis, did not show correlation with methylation status of the genes-under-study (data not shown).

DISCUSSION

Allison *et al*[16,17] first described and correctly interpreted the histopathological changes occurring in the distal esophagus of subjects suffering from GERD, and Paull *et al*[18], defined the histological subsets of the columnar lined esophagus. The subsets were then renamed the oxyntocardiac mucosa (formerly the fundic epithelium), cardiac mucosa (formerly the junctional epithelium), and intestinal metaplastic

Table 5 Clinical and histopathological data of patients depending on the presence of *Helicobacter pylori cagA*$^+$ infection by PCR, in esophageal biopsies

		Helicobacter pylori infection by PCR		*P* value
		Negative	Positive	
Age (yr)	≤ 40	16	9	0.390
	41-65	14	4	
	> 65	20	5	
Gender	Feminine	26	7	0.340
	Masculine	24	11	
Helicobacter pylori	Negative	38	8	0.014[1]
(HE)	Positive	12	10	
Sydney	Normal	38	8	0.014[1]
Classification:	Mild	11	6	
Helicobacter pylori	Moderate	1	2	
	Marked	0	2	
Sydney	Normal	23	5	0.028[1]
Classification:	Mild	25	8	
Neutrophils	Moderate	1	4	
	Marked	1	1	
Sydney	Mild	17	1	0.021[1]
Classification:	Moderate	30	13	
Mononuclear cells	Marked	3	4	

[1]Statistically significant. HE: Hematoxylin-eosin staining.

mucosa (formerly the specialized columnar epithelium) by Chandrasoma *et al*[19].

Although *H. pylori* infection of the gastric mucosa has been proposed as a beneficial factor for GERD and BE[20,21], nearly nothing has been published regarding the potential effects of *H. pylori* colonization on the esophageal non-specialized (gastric-type) metaplastic mucosa. To the best of our knowledge, this study is the first to examine the presence of *H. pylori cagA*$^+$ in columnar metaplastic mucosa of the esophagus and to correlate such bacterial presence with the appearance of early epigenetic events. We selected cases with gastric-type (non-specialized) columnar metaplasia of the esophagus because, in addition to being considered an early morphological change in the evolution of Barrett's esophagus[21], intestinal metaplastic cells are infrequently colonized by *H. pylori*[22]. Given the limited and random sampling of the columnar esophagus, we cannot yet rule out the presence of specialized columnar epithelium in areas other than those analyzed in the present study. However, our goal was to explore the methylation status of select genes and the relationship of methylation with *H. pylori cagA*$^+$ infection in gastric-type metaplastic mucosa of the esophagus.

The majority of these studies, however, have been planned and conducted employing normal settled esophageal mucosa or tissues displaying intestinal metaplasia and not considering non-specialized (gastric-type) metaplastic mucosa. In this study, we explored the methylation status of four genes, *DAPK* (proapoptotic; tumor suppressor), *THBS1* (angiogenesis inhibitor), *CDH1* (cell adhesion), and *p14ARF* (cell cycle regulator; tumor suppressor), in patients harboring gastric-type (non-specialized) columnar metaplasia of the esophagus. Of these, *DAPK* and *THBS1* methylation was significantly associated with *H. pylori cagA*$^+$ infection of the esophageal gastric-type metaplastic mucosa, whereas *CDH1* and *p14* genes methylation was not. In specialized columnar metaplasia of the esophagus (BE), *DAPK*, *CDH1*, and *p14* have been reported to be methylated in 50%, 8%, and 7%, respectively[23], whereas *THBS1* is infrequently methylated[6]. On the other hand, we did not find a statistically significant association between *H. pylori cagA* positive and methylation of any of these genes, in the gastric mucosa. Our findings could be interpreted as the result of the smaller sampling of the gastric mucosa than of the columnar lined esophagus, therefore, with a loss of any potential statistic association. Another plausible explanation is that metaplastic gastric mucosa, in the distal esophagus, is more susceptible to, or predisposes to, the methylation of certain genes than the normal settled gastric mucosa due to disturbances induced by the gastroesophageal reflux, in addition to the *H. pylori* infection.

It is widely recognized that *H. pylori* is able to colonize gastric mucosa along the entire gastrointestinal tract, including areas as proximal as the upper esophagus[24] and as distal as the rectum[25], as well as all sites in between, such as the duodenum[26] and Meckel's diverticulum[27]. Previously, colonization of gastric-type mucosa in BE was also described; however, its clinicopathological significance has been underestimated[5]. Recently, employing a rat experimental model of chronic gastroesophageal reflux, Liu *et al*[28] demonstrated that severity of inflammation and incidence of Barrett esophagus and esophageal adenocarcinoma are increased when *H. pylori* colonizes the esophagus. In the gastric mucosa, *H. pylori* causes a complex immune and inflammatory process that is largely determined by the virulence of strains carrying the cytotoxin-associated gene (*cag*) pathogenicity island (PAI)[29]. Thus, *H. pylori* is responsible for several molecular events that ultimately play a significant role in gastric carcinogenesis. Interestingly, *H. pylori* infection has been suggested as an initiator of gastric carcinogenesis by upregulation of DNA methyltransferase 3B (DNMT3B)[7], and it has been also reported to induce expression of DNMT1 and DNMT3A in gastrointestinal stromal tumors[30]; therefore, *H. pylori* DNMT-induced *de novo* methylation could promote aberrant CpG island methylation, thus increasing the risk of gastric cancer[31,32].

Dysregulation of *DAPK* is implicated in the development and progression of cancer through gene silencing. DAP kinase function is closely related with the *p*53-dependent pathway for apoptosis[33]. In the gastric mucosa, hypermethylation of the *DAPK* promoter has been associated with aging and chronic inflammation[11], as well as premalignant stages of gastric carcinoma[34]. Hypermethylation of the *DAPK* gene in noncancerous gastric and chronic gastritis mucosa has been associated

with the risk of gastric cancer and neutrophil infiltration activity, but not aging, in a *H. pylori*-infected population[10,12,35]. In the esophageal mucosa, decreases in DAPK protein expression correlate with the severity of reflux esophagitis and tumor progression in Barrett carcinogenesis[36].

Moreover, a field effect of *DAPK* has been detected in normal esophageal mucosa of patients with adenocarcinoma and Barrett esophagus[37]. Indeed, possible silencing of *DAPK* by methylation could be an early event in columnar metaplasia of the esophagus, and this silencing remains throughout the process of neoplastic transformation. The association between *DAPK* silencing and *H. pylori* infection has been previously reported in gastric mucosa[12]. It is noteworthy that *H. pylori* infection of the columnar mucosa could be exerting an additive effect on *DAPK* gene promoter methylation, in addition to the reflux.

On the other hand, *THBS1* is an inhibitor of angiogenesis and its expression is regulated by tumor-suppressor genes such as *p*53 and *Rb*. Additionally, *THBS1* possesses tumor suppressive properties *in vivo*. It has been demonstrated that *THBS1* methylation inactivates its expression in several normal and neoplastic cell lines[38].

DAPK and *THBS1* have been found to be methylated in the gastric mucosa in patients with diseases ranging from chronic gastritis to carcinoma, with a rising frequency of *DAPK* methylation encountered in advanced stages of carcinogenesis[36]. *THBS1* promoter methylation has been associated with *DAPK* methylation from early-onset sporadic gastric carcinoma[39]. In this way, our findings are in agreement with previous studies that demonstrated that the early steps of Barrett's progression may involve DNA methylation of genes linked with apoptosis and tumor suppressor properties, particularly *DAPK* and *THBS1*. Our findings are also consistent with those reported by Ferrández *et al*[40], who performed a large case-control study and observed that *H. pylori* CagA$^+$ infection does not reduce the risk of BE.

Finally, we are aware that our study has some limitations, regarding its retrospective design, the small number of patients under study, and the inevitable sampling bias due to the varied and random distribution of the histological changes among the columnar lined esophagus. Further prospective studies are warranted to adequately identify patients with GERD with higher risks for developing severe disorders including BE as well as adenocarcinoma and its precursor lesions.

In this study, we showed that CpG methylation occurs in non-specialized, gastric-type columnar metaplasia of the esophagus and is closely related to *H. pylori cagA*$^+$ infection. Given this effect on gastric-type metaplastic mucosa, a conscious search for *H. pylori* and its eradication may be essential for halting the early mechanisms potentially involved in BE development and BE-associated carcinogenesis, among subjects suffering from GERD.

COMMENTS

Background

Gastroesophageal reflux disease (GERD) is a highly prevalent condition among worldwide population, and gives rise to esophageal columnar metaplasia, among others. Nowadays, columnar metaplasia of the esophagus is classified into non-specialized (gastric-type) and specialized [intestinal-type; Barrett's esophagus (BE)] neoplastic transformation and progression in BE, are related to genetic and epigenetic events that ultimately favor abnormal expression of the genes responsible for the intrinsic control mechanisms regulating cellular proliferation and/or apoptosis.

Research frontiers

Current information suggests that intestinal metaplasia arises from gastric-type, non-specialized metaplasia. In the stomach, *Helicobacter pylori* (*H. pylori*) infection has been associated with methylation of multiple gene promoters involved in cell growth, differentiation and tumor suppression, as in chronic gastritis as in gastric cancer. In this study, the authors report the methylation of two genes and its relation to *H. pylori cagA* status, in gastric- type columnar metaplasia of the esophagus.

Innovations and breakthroughs

DNA methylation of the promoter regions of genes containing CpG islands is involved in the epigenetic regulation of gene expression (predominantly through gene repression), and it is an early event in the multistep process of esophageal carcinogenesis. The authors performed the first study to assess the methylation of some genes involved in neoplastic transformation and progression in several organs, and its correlation with *H. pylori cagA*$^+$ infection, in the esophageal gastric-type metaplastic mucosa.

Applications

This study provides evidence that CpG methylation occurs in non-specialized columnar metaplasia of the esophagus and is closely related to *H. pylori cagA*$^+$ infection. Given this effect on gastric-type metaplastic mucosa, a conscious search for *H. pylori* and its eradication may be essential for halting the early mechanisms potentially involved in BE development and BE-associated carcinogenesis, among subjects suffering from GERD.

Peer-review

Herrera-Goepfert *et al* explored gene methylation in esophageal columnar metaplasia, and correlated these findings with the status of *H. pylori cagA*$^+$. It is well written and contains information which readers may be interested. Because it is suggested that intestinal metaplasia in the esophagus arises from gastric-type metaplasia, authors should include intestinal metaplasia in the study.

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ORIGINAL ARTICLE

Retrospective Study

Correlation between *Helicobacter pylori*-associated gastric diseases and colorectal neoplasia

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Informed consent statement: Patients were not required to give informed consent to the study because the analysis used anonymous clinical data that were obtained after each patient agreed to treatment by written consent.

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Data sharing statement: No additional data are available.

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Abstract

AIM: To explore the correlation between *Helicobacter pylori* (*H. pylori*)-associated gastric diseases and colorectal neoplasia.

METHODS: Patients included in this study underwent a colonoscopy and esophago-gastro-duodenoscopy (EGD) along with histopathological measurement between March 2012 and March 2015 at Qi-Lu Hospital of Shandong University, who also had results of *H. pylori* detection. A total of 233 cases were selected. Demographic data, *H. pylori* infection status (including results of rapid urease tests and gastric mucosa pathological examinations) and histopathological examination results of gastric and colorectal mucosa were gathered and analyzed. The statistical analysis focused on the prevalence of colorectal neoplasms among patients with various histopathological categories of the stomach. ORs and their 95%CI were calculated to describe the strengths of the associations.

RESULTS: The incidence rates of colorectal adenoma without high-grade intraepithelial neoplasia (HGIEN) (OR = 2.400, 95%CI: 0.969-5.941), adenoma with HGIEN (5.333, 1.025-27.758) and adenocarcinoma (1.455, 0.382-5.543) were all higher for patients with *H. pylori*-associated gastritis than for those in the control group. The incidence rate of colorectal adenoma with HGIEN (3.218, 0.767-13.509) was higher in patients with intestinal metaplasia than in the control group, while the incidence rates of adenoma without HGIEN (0.874, 0.414-1.845) and adenocarcinoma (0.376, 0.096-1.470) were lower in the intestinal metaplasia group than in the control group. The incidence rate of colorectal adenoma without HGIEN (3.111, 1.248-7.753) was significantly higher in the gastric intraepithelial neoplasia group than in the control group, while the rates of adenoma with HGIEN (1.481, 0.138-15.941) and adenocarcinoma (2.020, 0.561-7.272) were higher in the gastric intraepithelial neoplasia group. Incidence rates of colorectal adenoma without HGIEN (1.067, 0.264-4.314), adenoma with HGIEN (2.667, 0.231-30.800) and adenocarcinoma (2.182, 0.450-10.585) were all higher in the gastric adenocarcinoma group than in the control group.

CONCLUSION: *H. pylori* infection as well as *H. pylori*-associated gastric diseases are risk factors for colorectal neoplasia.

Key words: *Helicobacter pylori*; *Helicobacter pylori*-associated gastric diseases; Colorectal neoplasia; Endoscopy with pathological biopsy; Chinese population

Core tip: Few studies have investigated the relationship between *Helicobacter pylori* (*H. pylori*)-associated gastric diseases and colorectal neoplasia. In particular, no such research on the Chinese population has been reported so far. To explore this correlation in the Chinese population, demographic data, *H. pylori* infection status and histopathological data of gastric and colorectal mucosa of 233 Chinese patients were gathered and analyzed. The results demonstrated that *H. pylori*-associated gastric diseases might increase the risk of colorectal neoplasia regardless of the number, size and location of the neoplasm. Therefore, we can assume that *H. pylori*-associated gastric diseases are potential risk factors for colorectal neoplasia in the Chinese population.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies worldwide. In China, the incidence and mortality rates of CRC have increased in recent years[1]. Due to the lack of specific clinical manifestations, the early diagnosis of CRC is relatively difficult, leading to the poor prognosis. Therefore, it is of great importance to elucidate the pathogenesis and risk factors of CRC, and develop relevant prevention and early detection strategies. During the development of CRC, the mucosa will progress from normal mucosa to adenoma first, and then to adenocarcinoma. This process provides the chance for early detection and intervention of CRC, and colorectal adenoma is considered the most important precancerous lesion for CRC. These two diseases, colorectal adenoma and CRC, are collectively called colorectal neoplasia.

It is believed that the development of colorectal neoplasia is associated with *Helicobacter pylori* (*H. pylori*) infection[2,3], although the pathophysiological mechanism underlying the correlation remains unclear. Most scholars believed that *H. pylori* might induce colorectal neoplasia by regulating the expression of serum gastrin[4,5]. Persistent *H. pylori* infection can lead to various gastric diseases, including gastritis, gastric intestinal metaplasia, gastric intraepithelial neoplasia and gastric adenocarcinoma. Chronic atrophic gastritis (CAG), which may progress to intestinal metaplasia, intraepithelial neoplasia and adenocarcinoma, can lead to decreased gastric acid secretion by extensive glandular atrophy. Serum gastrin level will increase accordingly through the negative feedback regulation, which shall then act as a trophic factor for colorectal mucosa. Therefore, different kinds of *H. pylori*-associated gastric diseases may be correlated with different levels of colorectal neoplasia depending on the serum gastrin level. Although several previous studies concluded that *H. pylori* seropositivity was associated with colorectal neoplasia[4,6-8], few have investigated the relationship between *H. pylori*-associated gastric diseases and colorectal neoplasia. In particular, no such research on the Chinese population has been reported so far.

In this research, we carried out a retrospective analysis of a database of 60501 Chinese patients who underwent esophago-gastro-duodenoscopy (EGD) and/or colonoscopy, trying to explore the possible correlation between *H. pylori*-associated gastric diseases and colorectal neoplasia.

MATERIALS AND METHODS

Patient selection

A total of 60501 Chinese patients underwent EGD and/or colonoscopy between March 2012 and March 2015 at Qi-Lu Hospital of Shandong University. Out

Table 1 Clinical indications for esophago-gastro-duodenoscopy and colonoscopy *n* (%)

Indication for EGD and colonoscopy	EGD	Colonoscopy
Abdominal discomfort	78 (33.5)	65 (27.9)
Diarrhea	26 (11.2)	47 (20.2)
Hematochezia	12 (5.2)	34 (14.6)
Weight loss	10 (4.3)	13 (5.6)
Others	58 (24.9)	39 (16.7)
Dyspepsia	33 (14.2)	
Reflux esophagitis	42 (18.0)	
Emesis	21 (9.0)	
Colorectal cancer screening		25 (10.7)
Polypectomy following-up		24 (10.3)

EGD: Esophago-gastro-duodenoscopy.

of those 60501 patients, those who had both EGD and complete colonoscopy (including the colonoscopy of the entire large intestine) were selected in the study. Histopathological results of gastric mucosa and colorectal mucosa as well as the results of *H. pylori* measurement were taken for all subjects. None of those patients in this study had a previous history of inflammatory bowel diseases (IBS), hereditary non-polyposis colorectal cancer (HNPCC) or familial adenomatous polyposis (FAP). None of them received *H. pylori* eradication therapy, gastrointestinal surgery, radiotherapy, chemotherapy, or other biotherapies targeting the cancer. No patients had a long-term drug use history. Based on the aforementioned criteria, a total of 233 patients were chosen.

Data collection

Demographic data, *H. pylori* infection status and histopathological results of gastric and colorectal mucosa were collected for all subjects. *H. pylori* infection status was determined by rapid urease test (RUT) and histopathological examination of gastric mucosa. *H. pylori* positivity was defined as results from one or both examinations were positive. EGD and colonoscopy were performed with EG-2990i electronic gastroscopes and EC-3890Fi electronic colonoscopes (Pentax, Tokyo, Japan), respectively. The location, number, and size of polyps were recorded during the colonoscopy.

All data were from existing records and personal identities were removed before the data were used in this study. Therefore, there was no need to obtain informed consent from patients.

Diagnostic criteria

Among the 233 patients, 159 (68.2%) had gastric antrum biopsies, 59 (25.3%) had gastric body and fundus biopsies, 29 (12.4%) had cardia biopsies, and 20 (8.6%) had multiple-site biopsies. The diagnostic criteria of the gastric biopsies were set according to the updated Sydney system[9]. Based on the sample size of this study, patients were divided into four groups according to their histopathological results of gastric mucosa: chronic gastritis group (including chronic non-atrophic gastritis and CAG), gastric intestinal metaplasia group, gastric intraepithelial neoplasia group and gastric adenocarcinoma group.

The following four histopathological categories were used for the colorectal mucosa: inflammation or non-adenomatous polyps (including hyperplastic polyps, inflammatory polyps, *etc.*), adenoma (including tubular adenoma, tubulovillous adenoma and villous adenoma) without high-grade intraepithelial neoplasia (HGIEN), adenoma with HGIEN and colorectal adenocarcinoma. Polyps were grouped based on their location such as rectum (including rectosigmoid junction), sigmoid colon, descending colon, transverse colon and ascending colon (including ileocecal junction). Polyps were also grouped based on the number: 1-3, 4-9 and > 10. Adenomas were grouped based on their size: 0-9 mm, 10-19 mm and > 20 mm.

Statistical analysis

The degree of correlations between *H. pylori*-associated gastric diseases and colorectal neoplasia was measured by ORs and their 95%CIs. χ^2 test was applied to calculate *P*-values. When the expected frequency was less than 5, Fisher's exact test was used to calculate *P*-values. *P*-values less than 0.05 were considered statistically significant. All statistical analyses were performed using Excel 2013 (Microsoft, Redmond, WA, United States) and SPSS 20.0 (SPSS, Chicago, IL, United States). The statistical methods of this study were reviewed by Dr. Jing Liu from Department of Epidemiology and Biostatistics, School of Public Health, Shandong University.

RESULTS

General characteristics of the study population

All 233 Chinese patients were between 16 and 89 years old, with the mean age at 56.85 ± 12.38 years. Of the 233 patients, 70.4% were males (164 patients), aged between 16 and 83 years with the mean at 56.69 ± 12.06 years, and 29.6% were females (69 patients), aged between 16 and 89 years with the mean at 57.22 ± 13.19 years. The clinical indications for EGD and colonoscopy are listed in Table 1.

Correlation between H. pylori-associated gastritis and colorectal neoplasia

H. pylori-associated gastritis is a gastric disease while the histopathological type was chronic gastritis (including chronic non-atrophic gastritis and CAG) with *H. pylori* infection (the infection status was determined by RUT and histopathological examination). Because no patient included in this research had completely normal histopathological results of gastric mucosa, patients with chronic gastritis and negative *H. pylori* were used as control group 1 in this research. For the

Table 2 Correlation between *Helicobacter pylori*-associated gastritis and colorectal neoplasia *n* (%)

Parameter	Total number of patients (*n* = 233)	*H. pylori*-associated gastritis	Control group 1	OR	95%CI	*P* value
Age	56.85	52.72 ± 11.37	56.42 ± 14.90	-	-	-
Male	164	27 (16.5)	48 (29.3)	1.000	-	-
Female	69	9 (13.0)	36 (52.2)	0.444	0.186-1.060	0.064
Control group 2	95	10 (10.5)	40 (42.1)	1.000	-	-
Adenoma without HGIEN	92	18 (19.6)	30 (32.6)	2.400	0.969-5.941	0.055
Adenoma with HGIEN	16	4 (25.0)	3 (18.8)	5.333	1.025-27.758	0.054
Adenocarcinoma	27	4 (14.8)	11 (40.7)	1.455	0.382-5.543	0.721
Polyp number						
1-3	106	16 (15.1)	36 (34.0)	1.778	0.716-4.414	0.212
4-9	46	10 (21.7)	14 (30.4)	2.857	0.983-8.306	0.049
10+	20	3 (15.0)	9 (45.0)	1.333	0.304-5.852	0.703
Adenoma size (mm)						
0-9	82	15 (18.3)	25 (30.5)	2.400	0.934-6.165	0.066
10-19	30	5 (16.7)	9 (30.0)	2.222	0.609-8.108	0.286
20+	16	4 (25.0)	7 (43.8)	2.286	0.558-9.366	0.256
Polyp location						
Rectum	79	14 (17.7)	33 (41.8)	1.697	0.667-4.315	0.264
Sigmoid colon	77	12 (15.6)	22 (28.6)	2.182	0.813-5.856	0.118
Descending colon	64	13 (20.3)	19 (29.7)	2.737	1.018-7.357	0.043
Transverse colon	55	11 (20.0)	17 (30.9)	2.588	0.927-7.230	0.065
Ascending colon	57	11 (19.3)	18 (31.6)	2.444	0.880-6.787	0.082

HGIEN: High-grade intraepithelial neoplasia; *H. pylori*: *Helicobacter pylori*.

same reason, patients with colorectal inflammation or non-adenomatous polyps were used as control group 2.

As shown in Table 2, compared to the patients in the control group 1, patients with *H. pylori*-associated gastritis were younger and had a higher rate of males. The incidence rates of colorectal adenoma without HGIEN, colorectal adenoma with HGIEN or colorectal adenocarcinoma were higher in the *H. pylori*-associated gastritis group than in the control group 1. The incidence rate of *H. pylori*-associated gastritis was much lower for patients in the control group 2 than for those in the other three groups (the colorectal adenoma group without HGIEN, the colorectal adenoma group with HGIEN and the colorectal adenocarcinoma group). The correlation between *H. pylori*-associated gastritis and colorectal neoplasms was the highest when the number of polyps was between 4 and 9 (OR = 2.857, 95%CI: 0.983-8.306, $P = 0.049$). In addition, the association of *H. pylori*-associated gastritis with colorectal neoplasms was independent of colorectal neoplasms size, with OR values greater than 1. The association was highest when the polyps were located at the descending colon (OR = 2.737, 95%CI: 1.018-7.357, $P = 0.043$).

Correlation between gastric intestinal metaplasia and colorectal neoplasia

There were more old people and males in the gastric intestinal metaplasia group than in the control group 1 (Table 3). The incidence rate of colorectal adenoma with HGIEN was higher in the gastric intestinal metaplasia group than in the control group 1, while the incidence rates of colorectal adenoma without HGIEN and colorectal adenocarcinoma were lower in the gastric intestinal metaplasia group. The inconsistency may be due to the small sample size, as there were only three patients with both gastric intestinal metaplasia and colorectal adenocarcinoma. Therefore, each individual case had a big impact on the OR value, leading to poor reliability of the final conclusion.

The association of gastric intestinal metaplasia with the number of colorectal neoplasms was also impacted by the small sample size, as the OR values varied considerably. The OR value was greater than 1 when the number of polyps was between 1 and 3 or between 4 and 9, while the OR value was smaller than 1 when the number of polyps was greater than 10. There were only five patients with both gastric intestinal metaplasia and more than 10 colorectal polyps. Also affected by the small sample size, the association of gastric intestinal metaplasia with the size of colorectal neoplasms was inconsistent. Since there was only one patient with both gastric intestinal metaplasia and colorectal adenoma larger than 20 mm, the analysis result could hardly be representative. This was also the case for the association of gastric intestinal metaplasia with the location of colorectal neoplasms.

Correlation between gastric intraepithelial neoplasia and colorectal neoplasia

Compared to patients in the control group 1, patients in the gastric intraepithelial neoplasia group were slightly older and had a significantly higher proportion of males ($P = 0.002$, Table 4). The incidence rate of colorectal adenoma without HGIEN was significantly higher in the gastric intraepithelial neoplasia group than in the control group 1 ($P = 0.013$). Similarly, the incidence rates of colorectal adenoma with HGIEN

Table 3 Correlation between gastric intestinal metaplasia and colorectal neoplasia *n* (%)

Parameter	Total number of patients (*n* = 233)	Gastric intestinal metaplasia	Control group 1	OR	95%CI	*P* value
Age	56.85	58.39 ± 7.34	56.42 ± 14.90	-	-	-
Male	164	44 (26.8)	48 (29.3)	1.000	-	-
Female	69	15 (21.7)	36 (52.2)	0.455	0.219-0.941	0.032
Control group 2	95	29 (30.5)	40 (42.1)	1.000	-	-
Adenoma without HGIEN	92	19 (20.7)	30 (32.6)	0.874	0.414-1.845	0.723
Adenoma with HGIEN	16	7 (43.8)	3 (18.8)	3.218	0.767-13.509	0.172
Adenocarcinoma	27	3 (11.1)	11 (40.7)	0.376	0.096-1.470	0.149
Polyp number						
1-3	106	30 (28.3)	36 (34.0)	1.149	0.582-2.270	0.688
4-9	46	12 (26.1)	14 (30.4)	1.182	0.477-2.929	0.717
10+	20	5 (25.0)	9 (45.0)	0.766	0.232-2.527	0.661
Adenoma size (mm)						
0-9	82	23 (28.0)	25 (30.5)	1.269	0.605-2.663	0.528
10-19	30	10 (33.3)	9 (30.0)	1.533	0.553-4.248	0.410
20+	16	1 (6.3)	7 (43.8)	0.197	0.023-1.690	0.140
Polyp location						
Rectum	79	19 (24.1)	33 (41.8)	0.794	0.379-1.664	0.541
Sigmoid colon	77	24 (31.2)	22 (28.6)	1.505	0.710-3.187	0.285
Descending colon	64	17 (26.6)	19 (29.7)	1.234	0.549-2.775	0.611
Transverse colon	55	14 (25.5)	17 (30.9)	1.136	0.484-2.668	0.770
Ascending colon	57	12 (21.1)	18 (31.6)	0.920	0.384-2.201	0.851

HGIEN: High-grade intraepithelial neoplasia.

Table 4 Correlation between gastric intraepithelial neoplasia and colorectal neoplasia *n* (%)

Parameter	Total number of patients (*n* = 233)	Gastric intraepithelial neoplasia	Control group 1	OR	95%CI	*P* value
Age	56.85	57.68 ± 11.24	56.42 ± 14.90	-	-	-
Male	164	32 (19.5)	48 (29.3)	1.000	-	-
Female	69	5 (7.2)	36 (52.2)	0.208	0.074-0.588	0.002
Control group 2	95	9 (9.5)	40 (42.1)	1.000	-	-
Adenoma without HGIEN	92	21 (22.8)	30 (32.6)	3.111	1.248-7.753	0.013
Adenoma with HGIEN	16	1 (6.3)	3 (18.8)	1.481	0.138-15.941	1.000
Adenocarcinoma	27	5 (18.5)	11 (40.7)	2.020	0.561-7.272	0.306
Polyp number						
1-3	106	17 (16.0)	36 (34.0)	2.099	0.832-5.293	0.112
4-9	46	10 (21.7)	14 (30.4)	3.175	1.071-9.413	0.033
10+	20	3 (15.0)	9 (45.0)	1.481	0.333-6.596	0.689
Adenoma size (mm)						
0-9	82	16 (19.5)	25 (30.5)	2.844	1.092-7.410	0.029
10-19	30	4 (13.3)	9 (30.0)	1.975	0.496-7.868	0.444
20+	16	3 (18.8)	7 (43.8)	1.905	0.411-8.829	0.409
Polyp location						
Rectum	79	12 (15.2)	33 (41.8)	1.616	0.607-4.304	0.335
Sigmoid colon	77	16 (20.8)	22 (28.6)	3.232	1.227-8.512	0.015
Descending colon	64	14 (21.9)	19 (29.7)	3.275	1.205-8.899	0.017
Transverse colon	55	13 (23.6)	17 (30.9)	3.399	1.223-9.443	0.016
Ascending colon	57	13 (22.8)	18 (31.6)	3.210	1.162-8.864	0.021

HGIEN: High-grade intraepithelial neoplasia.

and colorectal adenocarcinoma were also higher in the gastric intraepithelial neoplasia group. Gastric intraepithelial neoplasia was found more frequently in all other three groups (the colorectal adenoma group without HGIEN, the colorectal adenoma group with HGIEN and the colorectal adenocarcinoma group) than in the control group 2.

The association of gastric intraepithelial neoplasia with the number of colorectal neoplasia was similar to that of *H. pylori*-associated gastritis, as the OR values were all greater than 1. The association was the highest when the number of polyps was between 4 and 9 (OR = 3.175, 95%CI: 1.071-9.413, $P = 0.033$). The associations of gastric intraepithelial neoplasia with the size of colorectal neoplasia were also similar, with the association being strongest when the size of adenoma was 0-9 mm (OR = 2.844, 95%CI: 1.092-7.410, $P = 0.029$). Compared to that of *H.*

Table 5 Correlation between gastric adenocarcinoma and colorectal neoplasia *n* (%)

Parameter	Total number of patients (*n* = 233)	Gastric adenocarcinoma	Control group 1	OR	95%CI	*P* value
Age	56.85	62.36 ± 16.31	56.42 ± 14.90	-	-	-
Male	164	10 (6.1)	48 (29.3)	1.000	-	-
Female	69	4 (5.8)	36 (52.2)	0.533	0.155-1.838	0.314
Control group 2	95	5 (5.3)	40 (42.1)	1.000	-	-
Adenoma without HGIEN	92	4 (4.3)	30 (32.6)	1.067	0.264-4.314	1.000
Adenoma with HGIEN	16	1 (6.3)	3 (18.8)	2.667	0.231-30.800	0.418
Adenocarcinoma	27	3 (11.1)	11 (40.7)	2.182	0.450-10.585	0.379
Polyp number						
1-3	106	5 (4.7)	36 (34.0)	1.111	0.297-4.155	1.000
4-9	46	1 (2.2)	14 (30.4)	0.571	0.061-5.323	1.000
10+	20	0 (0.0)	9 (45.0)	-	-	-
Adenoma size (mm)						
0-9	82	3 (3.7)	25 (30.5)	0.960	0.211-4.372	1.000
10-19	30	2 (6.7)	9 (30.0)	1.778	0.296-10.671	0.614
20+	16	1 (6.3)	7 (43.8)	1.143	0.115-11.311	1.000
Polyp location						
Rectum	79	2 (2.5)	33 (41.8)	0.485	0.088-2.663	0.459
Sigmoid colon	77	3 (3.9)	22 (28.6)	1.091	0.238-5.003	1.000
Descending colon	64	1 (1.6)	19 (29.7)	0.421	0.046-3.859	0.657
Transverse colon	55	1 (1.8)	17 (30.9)	0.471	0.051-4.336	0.664
Ascending colon	57	3 (5.3)	18 (31.6)	1.333	0.287-6.192	0.702

HGIEN: High-grade intraepithelial neoplasia.

pylori-associated gastritis, the association of gastric intraepithelial neoplasia with the location of colorectal neoplasms was even stronger. The association was statistically significant when the polyps were located at the sigmoid colon, descending colon, transverse colon and ascending colon.

Correlation between gastric adenocarcinoma and colorectal neoplasia

Fourteen gastric adenocarcinoma patients were enrolled in the study (Table 5). Their average age (62.36 ± 16.31 years) was significantly higher than that of the control group 1 (56.42 ± 14.90 years). There was also a higher percentage of males in the gastric adenocarcinoma group than in the control group 1. The incidence rates of colorectal adenoma without HGIEN, colorectal adenoma with HGIEN and colorectal adenocarcinoma were higher in the gastric adenocarcinoma group than in the control group 1. However, the results were not statistically significant due to the small sample size. The incidence rate of gastric adenocarcinoma was higher in all three case groups than in the control group 2.

The relationship of gastric adenocarcinoma with the number of colorectal neoplasia varied widely because of the small sample size. There was no patient with both gastric adenocarcinoma and more than 10 colorectal polyps in this study. Also because of the small sample size, the association of gastric adenocarcinoma with the size of colorectal neoplasms was inconsistent, leading to low reliability. It was the same situation for the association of gastric adenocarcinoma with the location of colorectal neoplasia.

DISCUSSION

The first report that *H. pylori* infection might be associated with colorectal neoplasia (particularly colorectal adenomas) could be traced back to 1997[10]. Several previous studies have shown a positive correlation between *H. pylori* infection and colorectal neoplasia in different populations, such as African American[11], German[6,8], and Israelite[12]. However, other studies did not support the idea that *H. pylori* infection was associated with the development of colorectal neoplasia. For example, Strofilas *et al*[4] found that there was no significant difference of anti-*H. pylori* IgG antibodies between the colorectal cancer group and the control group in Greeks. A recent meta-analysis also failed to find a statistical association between *H. pylori* infection and colorectal neoplasia among the East Asian population[13]. In addition, such lack of association between *H. pylori* and colorectal adenomas was also reported in the United States Hispanic population[14]. Therefore, it was speculated that the relationship between *H. pylori* infection and colorectal neoplasia was race dependent[15], making it important to analyze data based on race.

Most previous studies used positive serology as the indicator of *H. pylori* infection[16-19], while others used the presence of *H. pylori*-associated gastritis as the indicator[20-22]. Yet very few studies used other *H. pylori*-associated gastric diseases as indicators during the investigation of the association of colorectal neoplasia with *H. pylori*-associated gastric diseases as well as their severity. In particular, to our knowledge, no such study has been performed on the Chinese population. Since Chinese represent more than one fifth of the

world total population, it is of great significance to investigate the relationship between *H. pylori*-associated gastric diseases and colorectal neoplasia in the Chinese population.

Our study demonstrated that *H. pylori*-associated gastric diseases might increase the risk of colorectal neoplasia regardless of the number, size and location of the neoplasm, although some results were not statistically significant as the sample size was too small. Generally we can assume that *H. pylori* infection as well as *H. pylori*-associated gastric diseases are potential risk factors for colorectal neoplasia.

Chinese population has a high prevalence of *H. pylori* infection and *H. pylori*-associated gastric diseases[23]. The incidence rate of colorectal adenocarcinoma is also high in China[24]. Early diagnosis of colorectal adenocarcinoma is relatively low even though early diagnosis is very important to lower the mortality[25]. Our research showed that people who had *H. pylori*-associated gastric diseases did have high risk of colorectal neoplasia. It is important to encourage patients with *H. pylori*-associated gastric diseases to undergo colonoscopy earlier and more frequently, to improve the early diagnostic rate of colorectal adenocarcinoma. In addition, people in the high-risk group should receive some interventions, such as lifestyle changes, which may lower the risk of developing cancer.

One advantage of this study was that RUT and histopathological results were used to determine the *H. pylori* infection status. As the gold standard for *H. pylori* infection diagnosis[26], histopathological examination can diagnose the *H. pylori* infection and pathologic changes of the stomach at the same time. Compared to serological tests that cannot differentiate existing infections from historical ones, RUT and histopathological tests diagnose only existing *H. pylori* infection. Such a distinction is vital since only existing *H. pylori* infection stimulates immune responses that can induce or perpetuate chronic inflammation in the gastrointestinal tract, and many malignancies are associated with epigenetic alterations induced by chronic inflammation[27,28].

How *H. pylori* infection increases the risk of colorectal neoplasia has not yet been elucidated. One common hypothesis is that hypergastrinemia induced by *H. pylori* infection contributes to the colorectal carcinogenesis, as high levels of gastrin can promote colorectal cell growth *in vitro* and increase colorectal cancer rates in animal models[29-31]. Since serum gastrin levels increase significantly as the healthy stomach progresses to malignancy[32], it could be inferred that the correlation between *H. pylori*-associated gastric diseases and colorectal neoplasia should be higher as the severity of the gastric lesions increases. However, our study showed that there was little association of the type of *H. pylori*-associated gastric diseases with colorectal neoplasia. This negative result might be due to the small sample size. As no gastrin level data was included in the study, it might also be because the gastrin levels were similar in different *H. pylori*-associated gastric diseases, as gastrin levels could be influenced by many factors. Yet other studies did show that gastrin levels were not related to colorectal neoplasia[6]. That being said, more studies are needed to clarify this issue. The relatively small sample size of our study also made it impossible to perform multivariate logistic analysis to eliminate possible confounding factors, restricting the research conclusion. Another limitation is that since all data were collected from the same center, some bias such as environmental factor might impact the results.

In conclusion, our study revealed that *H. pylori* infection and *H. pylori*-associated gastric diseases are potential risk factors of colorectal neoplasia. Early colonoscopy and interventions should be taken to reduce the risk of colorectal neoplasia for people with *H. pylori*-associated gastric diseases. Studies with larger sample size and multi-center data collection for Chinese population are needed to further clarify this association and to understand the underlying pathophysiological mechanism.

COMMENTS

Background

Colorectal cancer (CRC) is one of the most common malignancies worldwide. Colorectal adenoma is considered the most important precancerous lesion for CRC, and these two diseases are collectively called colorectal neoplasia. Accumulating evidence indicates that in addition to being a major risk factor of gastric cancer, *Helicobacter pylori* (*H. pylori*) infection is also associated with colorectal neoplasia. Although several previous studies concluded that *H. pylori* seropositivity was associated with colorectal neoplasia, few have investigated the relationship between *H. pylori*-associated gastric diseases and colorectal neoplasia. In particular, no such research on the Chinese population has been reported so far. In this study, we carried out a retrospective analysis of a database of 60501 Chinese patients who underwent esophago-gastro-duodenoscopy and/or colonoscopy, trying to explore the possible correlation between *H. pylori*-associated gastric diseases and colorectal neoplasia.

Research frontiers

Early diagnosis of colorectal cancer is very important to lower the mortality. Based on these results, it is critical for patients with *H. pylori*-associated gastric diseases to receive colonoscopy and interventions earlier to improve prognosis.

Innovations and breakthroughs

In this study, the authors investigated the possible correlation between *H. pylori*-associated gastric diseases and colorectal neoplasia in the Chinese population for the first time. Since the relationship between *H. pylori* infection and colorectal neoplasia was considered race dependent, these results supplemented the hypothesis that *H. pylori*-associated gastric diseases are potential risk factors of colorectal neoplasia with evidence from the Chinese population.

Applications

These results suggest that *H. pylori* infection and *H. pylori*-associated gastric diseases are potential risk factors of colorectal neoplasia, encouraging people in the high-risk group to receive colonoscopy and some interventions earlier and more frequently, thus improve the early diagnostic rate of colorectal adenocarcinoma.

Peer-review

It is an interesting study, but includes too few subjects to have such a conclusion. It is in the text that *H. pylori* infection may affect colorectal neoplasm formation differently in different races. And this study reported for the first time how *H. pylori*-associated gastric diseases are correlated with colorectal neoplasm in the Chinese people, representing its innovation.

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ORIGINAL ARTICLE

Observational Study

Changes in patients' symptoms and gastric emptying after *Helicobacter pylori* treatment

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Abstract

AIM: To investigate the changes in clinical symptoms and gastric emptying and their association in functional dyspepsia (FD) patients.

METHODS: Seventy FD patients were enrolled and divided into 2 groups *Helicobacter pylori* (*H. pylori*)-negative group (28 patients), and *H. pylori*-positive group (42 patients). Patients in the *H. pylori*-positive group were further randomly divided into groups: *H. pylori*-treatment group (21 patients) and conventional treatment group (21 patients). Seventy two healthy subjects were selected as the control group. The proximal and distal stomach area was measured by ultrasound immediately after patients took the test meal, and at 20, 40, 60 and 90 min; then, gastric half-emptying time was calculated. The incidence of symptoms and gastric half-emptying time between the FD and control groups were compared. The *H. pylori*-negative and conventional treatment groups were given

conventional treatment: domperidone 0.6 mg/(kg/d) for 1 mo. The *H. pylori*-treatment group was given *H. pylori* eradication treatment + conventional treatment: lansoprazole 30 mg once daily, clarithromycin 0.5 g twice daily and amoxicillin 1.0 g twice daily for 1 wk, then domperidone 0.6 mg/(kg/d) for 1 mo. The incidence of symptoms and gastric emptying were compared between the FD and control groups. The relationship between dyspeptic symptoms and gastric half-emptying time in the FD and control groups were analyzed. Then total symptom scores before and after treatment and gastric half-emptying time were compared among the 3 groups.

RESULTS: The incidence of abdominal pain, epigastric burning sensation, abdominal distension, nausea, belching, and early satiety symptoms in the FD group were significantly higher than in the control group (50.0% *vs* 20.8%; 37.1% *vs* 12.5%; 78.6% *vs* 44.4%; 45.7% *vs* 22.2%; 52.9% *vs* 15.3%; 57.1% *vs* 19.4%; all $P < 0.05$). The gastric half-emptying times of the proximal end, distal end, and the whole stomach in the FD group were slower than in the control group (93.7 ± 26.2 *vs* 72.0 ± 14.3; 102.2 ± 26.4 *vs* 87.5 ± 18.2; 102.1 ± 28.6 *vs* 78.3 ± 14.1; all $P < 0.05$). Abdominal distension, belching and early satiety had an effect on distal gastric half-emptying time ($P < 0.05$). Abdominal distension and abdominal pain had an effect on the gastric half-emptying time of the whole stomach ($P < 0.05$). All were risk factors (odds ratio > 1). The total symptom score of the 3 groups after treatment was lower than before treatment ($P < 0.05$). Total symptom scores after treatment in the *H. pylori*-treatment group and *H. pylori*-negative group were lower than in the conventional treatment group (5.15 ± 2.27 *vs* 7.02 ± 3.04, 4.93 ± 3.22 *vs* 7.02 ± 3.04, All $P < 0.05$). The gastric half-emptying times of the proximal end, distal end, and the whole stomach in the *H. pylori*-negative and *H. pylori*-treatment groups were shorter than in the conventional treatment group ($P < 0.05$).

CONCLUSION: FD patients have delayed gastric emptying. *H. pylori* infection treatment helps to improve symptoms of dyspepsia and is a reasonable choice for treatment in clinical practice.

Key words: Functional dyspepsia; Gastric emptying; Ultrasound

Core tip: Stomach half-emptying time was determined in *Helicobacter pylori* (*H. pylori*) patients and healthy controls. The half-emptying times at the proximal end, distal end, and the whole stomach in the functional dyspepsia (FD) group were slower than in the control group. Total symptom scores in the *H. pylori*-treatment group and *H. pylori*-negative group were lower than in the conventional treatment group after treatment. Patients with FD have delayed gastric emptying. Treatment of *H. pylori* infection helps to improve symptoms of dyspepsia and is a reasonable choice for therapy in clinical practice.

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INTRODUCTION

Functional dyspepsia (FD) is the most common functional gastrointestinal disorder, but its etiology remains unclear[1-3]. However, it is generally believed that an abnormality in gastric motility is an important factor[4]. Patients with FD often appear to have abdominal distension, belching, nausea, and other symptoms of dyspepsia. In severe cases, it has a major impact on daily life, and treatment is required to alleviate the symptoms[5-7]. FD is associated with a variety of factors that include gastrointestinal motility disorders, gastrointestinal hormone secretion abnormalities, or *Helicobacter pylori* (*H. pylori*) infection[8]. Among them, *H. pylori* infection probably induces symptoms by increasing the sensitivity to mechanical distension or increasing gastric acid secretion[9]. Clinically, gastric motility drug treatment is primarily given to these patients. However, whether there is a need for eradication therapy for *H. pylori* in FD patients remains controversial[10]. Furthermore, determination of gastric emptying by ultrasound is convenient for observation, has economic advantages, is easy for patients, and is suitable for widespread or repeated use[11,12]. Therefore, this study compared symptoms of dyspepsia, gastric emptying time, and other indicators before and after *H. pylori* treatment in patients with FD in our hospital, and in healthy volunteers. The relationship between the symptoms of dyspepsia and gastric half-emptying time were observed in FD patients, and whether *H. pylori* treatment could alleviate the symptoms was investigated, with the aim of providing a basis for the clinical treatment.

MATERIALS AND METHODS

Study population

A total of 70 adult FD patients admitted to our hospital from January 2013 to March 2015 were included in this study as the FD group, and were divided into 2 groups: *H. pylori*-negative group ($n = 28$) and *H. pylori*-positive group ($n = 42$). The *H. pylori*-positive group was further randomly divided into 2 groups: an *H. pylori* treatment group and a conventional treatment group ($n = 21$ each group). Patients with

Table 1 Comparison of baseline characteristics between the functional dyspepsia and control groups

Group	Age	Sex (male/female)	BMI (kg/m^2)	Duration of disease (yr)
FD group (n = 70)	44.82 ± 13.41	27/43	21.38 ± 3.87	2.01 ± 1.32
Control group (n = 72)	40.70 ± 6.39	22/48	21.20 ± 2.95	1.89 ± 1.73
t-test value	t = 2.347	χ^2 = 0.785	t = 0.312	t = 0.464
P value	0.020	0.376	0.755	0.644
H. pylori therapeutic group	42.73 ± 11.97	10/18	20.98 ± 3.27	2.10 ± 1.12
Conventional therapeutic group	43.79 ± 14.28	9/12	22.08 ± 2.96	1.97 ± 1.28
H. pylori-negative group	45.82 ± 12.83	8/13	21.16 ± 3.64	1.95 ± 1.31
t-test value	F = 2.191	F = 2.315	F = 1.923	F = 1.872
P value	0.266	0.221	0.397	0.426

BMI: Body mass index; FD: Functional dyspepsia; *H. pylori*: *Helicobacter pylori*.

FD met the following criteria[13]: (1) the Rome Ⅲ diagnostic criteria of FD; (2) duration of disease 1-3 years, with no gastrointestinal motility or *H. pylori* drug treatment in the previous month; (3) had not been treated with systematic FD or *H. pylori* drugs; and (4) underwent pathological examination by gastroscopy and a ^{13}C-urea breath test. *H. pylori*- positive patients were required to have 2 positive checks, and excluded single-positive patients. In addition, 72 healthy adults were selected from the Medical center as the control group. None of the subjects had the following exclusion criteria[13]: (1) organic lesions in the stomach and duodenum revealed by endoscopic examinations; (2) history of gastrointestinal surgery; (3) diabetes or connective tissue diseases; and (4) long-term smoking or alcoholism. Age and other characteristics were similar between the FD group and control group (P > 0.05, Table 1).

Methods

A GE Voluson E8 ultrasound with a C1-5 transducer was used for examination of all subjects by an experienced sonographer. Subjects were not allowed to drink and eat 12 h before the ultrasound examination, and the empty state of the stomach was confirmed before examination[14]. Patients were asked to finish a 500 mL standard test meal within 4-5 min (500 mL of 80 g black sesame paste in boiled water, cooled to about 25 ℃; about 1960 kJ)[15]. With patients in a sitting position, the abdomen between the xiphoid and navel was first scanned by an ultrasound 4C1 convex array probe The "figure-of-eight-like" double ring sign (Figure 1) junction is the angulus, which is the boundary between the proximal end and distal ends of the stomach. The proximal and distal stomach areas were measured immediately after patients took the test meal and at 20, 40, 60, and 90 min thereafter, and gastric half-emptying time was obtained by computer analysis.

FD patients in the *H. pylori*-negative group and conventional treatment group were given domperidone 0.6 mg/kg/d 30 min before a meal for 1 mo; while patients in the *H. pylori* treatment group took a combination of 3 drugs as eradication therapy for *H. pylori*, including lansoprazole tablets 30 mg once daily, clarithromycin tablets 0.5 g twice daily, and amoxicillin 1.0 g twice daily for 1 wk. Confirmation of *H. pylori* eradication was performed with a gastroscope and ^{13}C-urea breath test. Then domperidone 0.6 mg/kg per day) was given 30 min before a meal for 1 mo. Dyspepsia symptoms pre-and-post treatment were scored in all these groups. Gastric half-emptying time was determined after the end of treatment.

Evaluation index

Dyspepsia symptoms of all subjects were statistically compared, and the correlation with gastric half-emptying time was analyzed. Total dyspepsia symptom scores before and after treatment were compared and analyzed among the 3 groups of patients with FD. Symptom scores for abdominal pain, epigastric burning sensation, abdominal distension, nausea, belching, vomiting, and early satiety were scored according to the severity of symptoms: 0, asymptomatic; 1, mild (between asymptomatic and moderate); 2, moderate (symptoms that can be tolerated); 3, severe (symptoms that are intolerable and have a serious impact on daily life)[16,17]. Then, gastric half-emptying time after treatment was compared among the 3 groups of patients with FD.

Statistical analysis

SPSS 20.0 was used to analyze all data (IBM Corp., Armonk, NY, United States). P < 0.05 was considered statistically significant. The incidence of epigastric abdominal pain, epigastric burning sensation, abdominal distension, nausea, belching, vomiting, and early satiety symptoms was compared between the FD group and control group using the χ^2 test. Gastric half-emptying time: Gastric half-emptying time of the proximal end, distal end, and the whole gastric region was compared between the FD group and control group using the t-test. The relationship between dyspepsia symptoms and gastric half-emptying was determined using logistic regression analysis, with gastric half-emptying time as the dependent variable and the symptoms of dyspepsia as the independent variables. Total symptom scores before and after treat-

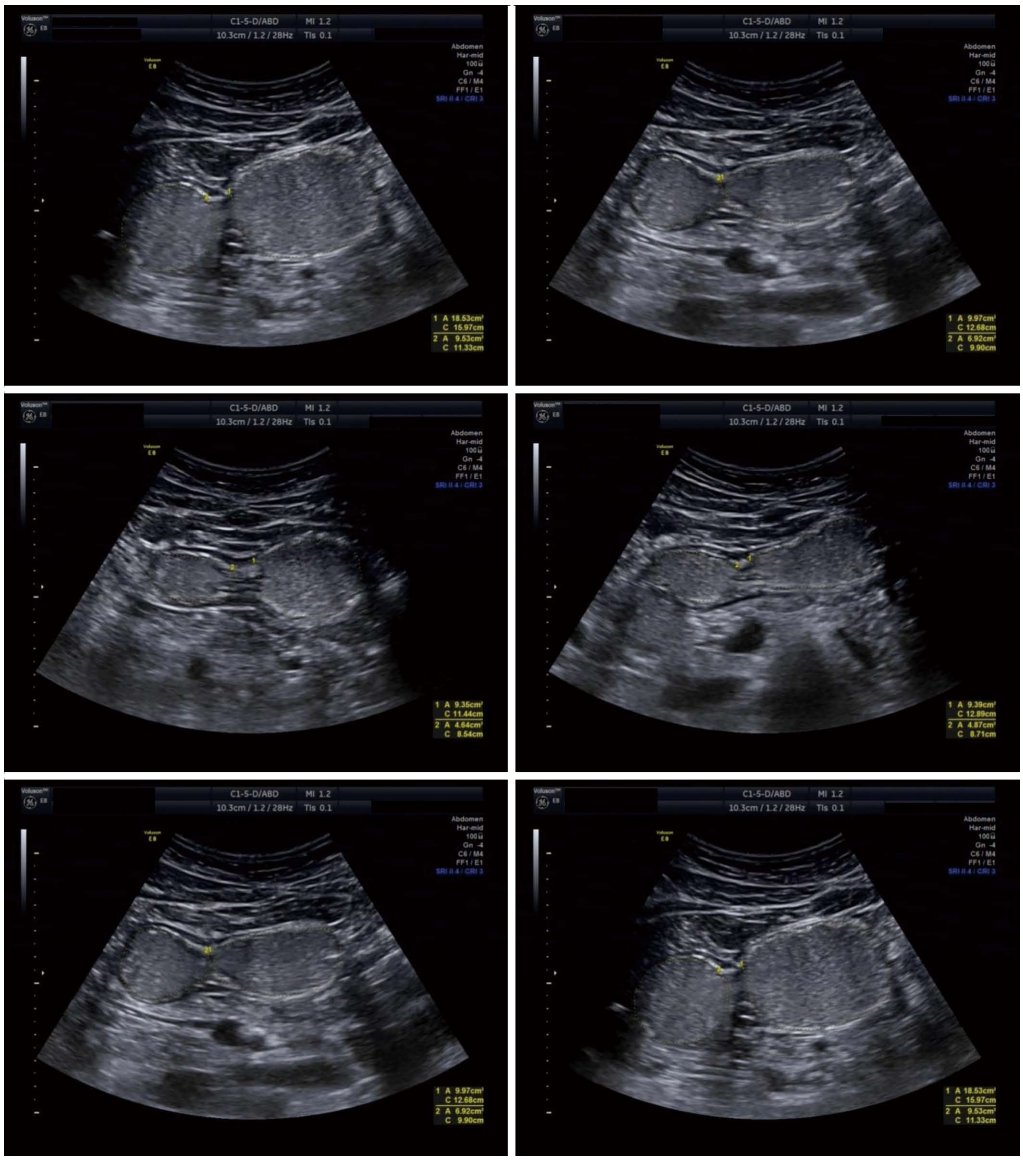

Figure 1 Gastric "figure-of-eight-like" double ring sign in different patients.

ment in the 3 groups of FD patients were compared by one-way analysis of variance (ANOVA test). The SNK-*Q* test was then performed for paired comparisons when the difference between groups was statistically significant. The gastric half-emptying times at the proximal end, distal end, and the whole gastric region after treatment among the 3 groups of patients were compared by ANOVA, followed by the SNK-*Q* test for paired comparisons.

RESULTS

Comparison of dyspepsia symptoms

The incidence of abdominal pain, epigastric burning sensation, abdominal distension, nausea, belching, and early satiety symptoms was significantly higher in patients in the FD group compared with the control group ($P < 0.05$). However, there was no significant difference in the incidence of vomiting between the 2 groups ($\chi^2 = 1.624$, $P = 0.203$), as shown in Table 2.

Comparison of gastric half-emptying conditions

Gastric half-emptying times at the proximal end, distal end, and the whole gastric region were significantly slower in the FD group than in the control group ($P < 0.05$, Table 3). As observed from the gastric emptying curve for the 2 groups, gastric contents in the proximal end presented a gradual downward trend in the control group, which slowed down between 40 min and 60 min. However, the rate of decline was slower in the FD group compared with the control group, and the decline between 40 and 60 min time points was significantly slower, presenting a plateau phase. The control group continued to present a slow downward trend in the gastric emptying curve at the distal end. However, the curve presented an upward and a downward trend in the FD group within the first 20 min after eating, and was slower than that in the

Table 2 Incidence of dyspeptic symptoms in functional dyspepsia and control groups *n* (%)

Groups	Abdominal pain	Epigastric burning sensation	Abdominal distension	Nausea	Belching	Vomiting	Early satiety
FD group (n = 70)	35 (50.0)	26 (37.1)	55 (78.6)	32 (45.7)	37 (52.9)	11 (15.7)	40 (57.1)
Control (n = 72)	15 (20.8)	9 (12.5)	32 (44.4)	16 (22.2)	5 (15.3)	7 (9.7)	14 (19.4)
χ^2 value	18.642	16.224	24.699	12.314	31.456	1.624	30.087
P value	0.000	0.000	0.000	0.000	0.000	0.203	0.000

FD: Functional dyspepsia.

Table 3 Comparison of gastric half-emptying time between functional dyspepsia and control groups

Groups	Gastric half-emptying time at the proximal end (min)	Gastric half-emptying time at the distal end (min)	Gastric half-emptying time at the whole gastric region (min)
FD group (n = 70)	93.7 ± 26.2	102.2 ± 26.4	102.1 ± 28.6
Control group (n = 72)	72.0 ± 14.3	87.5 ± 18.2	78.3 ± 14.1
t value	6.149	3.782	6.316
P value	0.000	0.000	0.000

FD: Functional dyspepsia.

control group. In the gastric emptying curve of the whole gastric region, the control group continued to present a slow downward trend. However, the curve for the FD group declined at different rates (Figure 2).

Relationship between symptoms of dyspepsia and gastric half-emptying time

There was no significant association of any of the symptoms with prolonged gastric half-emptying time at the proximal end (P > 0.05). However, abdominal distension, belching, and early satiety was associated with abdominal distension at the distal end (P < 0.05), and were risk factors for delayed gastric half-emptying time at the distal end (OR > 1). Abdominal distension and abdominal pain were associated with gastric half-emptying time of the whole gastric region (P < 0.05), and were risk factors for delayed gastric half-emptying of the whole gastric region (OR > 1, Table 4).

Total score of patients with symptoms of dyspepsia occurring before and after treatment

There was no statistically significant difference in total patient symptom scores before treatment among the 3 groups (F = 3.021, P = 0.291). However, the scores were lower after treatment than before treatment, and the difference was statistically significant (P < 0.05). There was a statistically significant difference in scores after treatment among the 3 groups (F = 3.162, P = 0.014). Pairwise comparisons showed total symptom scores after treatment were significantly lower in the *H. pylori* treatment group and *H. pylori*-negative group than in the conventional treatment group (*H. pylori* treatment group: Q = 2.259, P = 0.029; *H. pylori*-negative group: Q = 2.163, P = 0.037); however, there was no significant difference between the *H. pylori* treatment group and *H. pylori*-negative group (Q = 0.270, P = 0.791; Table 5).

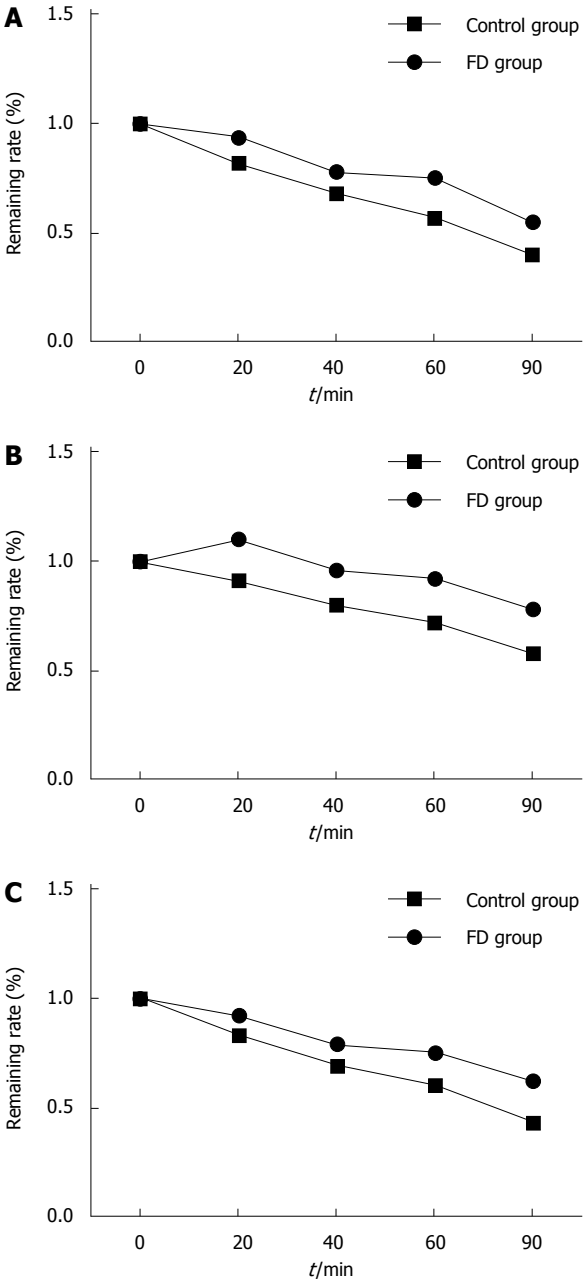

Figure 2 Gastric emptying curves. A: Proximal end; B: Distal end; C: Whole gastric region.

Table 4 Logistic regression analysis of symptoms of dyspepsia and gastric half-emptying time

Influential factors	Prolonged gastric half-emptying time	β	SE	Wald value	OR	95%CI	*P* value
Abdominal pain	Proximal	0.255	0.322	5.743	1.291	0.687-2.426	0.791
	Distal	0.123	0.429	6.841	1.131	0.488-2.622	0.387
	Whole	0.780	0.117	15.935	2.182	1.735-2.744	0.008
Epigastric burning sensation	Proximal	0.653	0.412	6.472	1.921	0.857-4.308	0.391
	Distal	-0.272	0.366	5.937	0.762	0.372-1.561	0.752
	Whole	-0.183	0.621	6.935	0.833	0.247-2.814	0.326
Abdominal distension	Proximal	-0.451	0.429	7.299	0.637	0.275-1.477	0.261
	Distal	0.600	0.128	15.643	1.823	1.419-2.343	0.016
	Whole	0.273	0.118	14.984	1.314	1.043-1.656	0.021
Nausea	Proximal	0.545	0.529	8.327	1.725	0.612-4.865	0.134
	Distal	0.205	0.326	7.565	1.227	0.648-2.325	0.142
	Whole	0.699	0.762	7.418	2.011	0.452-8.954	0.221
Belching	Proximal	0.023	0.376	7.488	1.023	0.490-2.138	0.172
	Distal	0.745	0.223	12.473	2.106	1.360-3.260	0.031
	Whole	-0.583	0.515	9.304	0.558	0.203-1.531	0.086
Early satiety	Proximal	-0.467	0.718	8.471	0.627	0.153-2.561	0.096
	Distal	0.461	0.202	13.845	1.585	1.067-2.355	0.026
	Whole	-0.028	0.486	8.737	0.972	0.375-2.520	0.093

Table 5 Comparison of total symptom scores of patients before and after treatment

Groups	Before treatment	After treatment	*Q* value	*P* value
H. pylori treatment group (n = 21)	10.14 ± 4.02	5.15 ± 2.27	4.953	0.000
Conventional treatment group (n = 21)	11.01 ± 3.92	7.02 ± 3.04	3.686	0.001
H. pylori-negative group (n = 28)	11.61 ± 4.81	4.93 ± 3.22	6.107	0.000

H. pylori: *Helicobacter pylori*.

Table 6 Comparison of gastric half-emptying time (min) of patients after treatment

Groups	Gastric half-emptying time at the proximal end	Gastric half-emptying time at the distal end	Gastric half-emptying time at whole gastric region
(1) *H. pylori* treatment group (n = 21)	74.0 ± 12.4	87.7 ± 13.4	80.3 ± 14.4
(2) Conventional treatment group (n = 21)	83.1 ± 15.8	97.5 ± 15.1	91.9 ± 17.2
(3) *H. pylori*-negative group (n = 28)	73.6 ± 11.7	88.3 ± 15.4	79.8 ± 15.9
F value	3.211	3.143	3.188
P value	0.004	0.019	0.007
(1):(2) *Q* value	2.076	2.225	2.37
P value	0.044	0.032	0.023
(1):(3) *Q* value	0.115	0.143	0.107
P value	0.909	0.887	0.915
(3):(2) *Q* value	2.317	2.087	2.516
P value	0.025	0.042	0.015

H. pylori: *Helicobacter pylori*.

Gastric half-emptying time after treatment

There was a statistically significant difference in gastric half-emptying time at the proximal end, distal end, and whole gastric region after treatment among the 3 groups ($P < 0.05$). Pairwise comparisons showed that gastric half-emptying times at the proximal end, distal end, and whole gastric region were significantly shorter in the *H. pylori*-negative group and *H. pylori* treatment group compared with the conventional treatment group ($P < 0.05$); while there was no significant difference between the *H. pylori* treatment group and *H. pylori*-negative group ($P > 0.05$, Table 6).

DISCUSSION

In patients with FD, although there is no organic disease, belching, nausea, abdominal pain, and other symptoms of dyspepsia can continue for more than 6 mo, and if symptoms persist for 3 mo or more, there is a greater impact on the quality of life[17-20]. The incidence of FD is about 20%, and is mostly caused by gastric motility disorders, including abnormal gastric emptying, stomach discomfort from reduced capacity, and gastric electrical rhythm abnormalities[21-24]. The stomach can be divided into 2 regions at the angulus, and the proximal and distal regions function differently to some extent. The proximal region mainly functions to receive and store food, and control liquid emptying. The distal region may conduct peristalsis to grind the food and mix it with the gastric juice[25-29]. Observation of gastric emptying by ultrasound is a simple method, does not cause injury, and is easily accepted by patients. Therefore, gastric emptying studies by ultrasound observation enable the comparison of the relationship between the symptoms of patients with FD. In this study, a comparative analysis was performed for gastric emptying and various common FD symptoms in the proximal end, distal end and full stomach. *H. pylori* infection affects the endocrine aspects of the smooth muscle in the human gastro-

intestinal tract, and this is generally considered to have a significant impact on the symptoms in patients with FD[30-32]. Therefore, in this study, *H. pylori*-positive and -negative symptoms and gastric emptying between healthy subjects and patients with FD were compared in order to observe the effects of *H. pylori* infection in FD patients.

The study revealed that the incidence of symptoms in the FD group, except vomiting, was high compared with the control group. Gastric half-emptying time at the proximal end, distal end, and the whole gastric region was slower in the FD group than in the control group. Abdominal distension, belching, and early satiety were associated with gastric half-emptying time at the distal end. Abdominal distension and abdominal pain were associated with gastric half-emptying time of the whole gastric region. Total symptom scores of patients in the 3 groups decreased after treatment. In the *H. pylori*-negative group and *H. pylori* treatment group, total symptom scores after treatment were lower compared with the conventional treatment group; and gastric half-emptying times at the proximal end, distal end, and the whole gastric region were shorter than in the conventional treatment group. However, there was no difference between the *H. pylori* treatment group and *H. pylori*-negative group. It can be observed that delayed gastric emptying and *H. pylori* infection have an important impact on the occurrence of symptoms in FD patients. As observed from the gastric emptying curve at the distal end after eating, the amount of food inside the stomach at the distal end increased and slowly declined. It is considered that this is because the proximal end of the stomach suffers from receptive dysfunction for food. When food increases, relaxation is delayed, resulting in regurgitation or the rapid discharge of food into the distal end, causing FD patients to have early satiety, abdominal distension, and other symptoms[33-36]. In addition, the abnormal distribution of food at the proximal and distal end of the stomach further affects the emptying of food, resulting in the occurrence of symptoms of dyspepsia[37].

After administration of drugs promoting gastric motility to patients, the clinical symptoms of FD patients were alleviated regardless of whether *H. pylori* infection was present or not. As observed, abnormal gastric motility is an important reason for the occurrence of FD symptoms. *H. pylori* eradication treatment to improve symptoms and gastric emptying of *H. pylori*-positive patients is better than no *H. pylori* eradication treatment. It has been considered that *H. pylori* participate in the occurrence of FD symptoms through a variety of mechanisms[9]. This includes *H. pylori* infections in the gastrointestinal tract causing an increase in mechanical expansion sensitivity[38,39]. *H. pylori* infection can directly lead to increased gastric acid secretion or promote gastric acid secretion by increasing gastrin, leading to abdominal pain, epigastric burning sensations, and other symptoms[40]. *H. pylori* infection can cause gastrointestinal hormone secretion disorders, such as increased somatostatin and cholecystokinin, leading to an increase in the incidence of symptoms in patients with FD[41-44]. *H. pylori* infection affects gastric emptying, and influencing factors include increased release of leukotrienes[45,46], or nitric oxide and other substances. This leads to gastrointestinal smooth muscle relaxation and delayed gastric emptying, or an increase in 5-HT and other substances affecting gastrointestinal smooth muscle contraction, resulting in gastrointestinal tract motility disorders[47-50].

As the amount of samples collected in this study was small and it was a single center study, the relationship between symptoms of dyspepsia in patients with FD, gastrointestinal tract motility disorders, and *H. pylori* infections requires further larger scale investigations to further determine the pathophysiological mechanisms in order to provide good guidance for clinical diagnosis and treatment of patients with FD.

In summary, gastric emptying is delayed to some extent in patients with FD. For patients infected with *H. pylori*, *H. pylori* eradication treatment helps to improve dyspepsia symptoms. This may be a reasonable choice for therapy in clinical practice.

COMMENTS

Background

Functional dyspepsia (FD) is the most common functional gastrointestinal disorder, but the etiology remains unclear. Patients with FD often appear to have abdominal distension, belching, nausea, and other symptoms of dyspepsia. In severe cases, it seriously impacts their daily life. FD is related to a variety of causes that include gastrointestinal motility disorders, gastrointestinal hormone secretion abnormalities, or *Helicobacter pylori* (*H. pylori*) infection. However whether there is a need for eradication therapy for *H. pylori* in FD patients remains controversial. Therefore, the study investigated the relationship between symptoms of dyspepsia and gastric half-emptying time in FD patients, and whether *H. pylori* treatment could alleviate the symptoms, to provide a basis for clinical treatment.

Research frontiers

In recent years, FD incidence has gradually increased and its cause is unknown. Studies have reported that *H. pylori* infection can cause gastrointestinal hormone secretion disorders such as increased somatostatin and cholecystokinin secretion, leading to a corresponding increase in the incidence of symptoms in patients with FD. The symptoms of FD are mostly caused by gastric motility disorders, including abnormal gastric emptying, stomach discomfort from reduced capacity, and gastric electrical rhythm abnormalities. The use of ultrasound to observe gastric emptying, and compare symptoms score, is a simple and effective method.

Innovations and breakthroughs

FD is mostly caused by gastric motility disorders including abnormal gastric emptying. Gastric emptying observation by ultrasound is a simple and effective method, does not cause injury, and is easily accepted by patients. Using symptom scoring for a variety of FD symptoms, a more comprehensive evaluation of symptoms can be made.

Applications

This study demonstrated that patients with FD have delayed gastric emptying. *H. pylori* infection treatment helps to improve symptoms of dyspepsia. FD is closely associated with abnormal gastric emptying and *H. pylori* infection. This provides a basis for clinical treatment with gastrointestinal motility drugs and *H.*

pylori eradication therapy in FD patients.

Peer-review

This study compared and analyzed the gastric emptying and symptoms between FD patients and healthy people; *H. pylori* positive group, *H. pylori*-negative group and the conventional treatment. It demonstrates that patients with FD have delayed gastric emptying. *H. pylori* infection treatment helps to improve symptoms of dyspepsia. Provide a reliable basis to applications gastrointestinal drugs and drugs to cure *H. pylori* for the treatment of patients with *H. pylori*.

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Calcium supplementation for the prevention of colorectal adenomas: A systematic review and meta-analysis of randomized controlled trials

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Abstract

AIM: To determine the efficacy of calcium supplementation in reducing the recurrence of colorectal adenomas.

METHODS: We conducted a systematic review and meta-analysis of published studies. We searched PubMed, Scopus, the Cochrane Library, the WHO International Clinical Trials Registry Platform, and the ClinicalTrials.gov website, through December 2015. Randomized, placebo-controlled trials assessing supplemental calcium intake for the prevention of recurrence of adenomas were eligible for inclusion. Two reviewers independently selected studies based on predefined criteria, extracted data and outcomes (recurrence of colorectal adenomas, and advanced or "high-risk" adenomas), and rated each trial's risk-of-bias. Between-study heterogeneity was assessed, and pooled risk ratio (RR) estimates with their 95% confidence intervals (95%CI) were calculated using fixed- and random-effects models. To express the treatment effect in clinical terms, we calculated the number needed to treat (NNT) to prevent one adenoma recurrence. We also assessed the quality of evidence using GRADE.

RESULTS: Four randomized, placebo-controlled trials met the eligibility criteria and were included. Daily doses of elemental calcium ranged from 1200 to 2000 mg, while the duration of treatment and follow-up of participants ranged from 36 to 60 mo. Synthesis of intention-to-treat data, for participants who had undergone follow-up colonoscopies, indicated a modest protective effect of calcium in prevention of adenomas (fixed-effects, RR = 0.89, 95%CI: 0.82-0.96; random-effects, RR = 0.87, 95%CI: 0.77-0.98; high quality of evidence). The NNT was 20 (95%CI: 12-61) to prevent one colorectal adenoma recurrence within a period of 3 to 5 years. On the other hand, the association between calcium treatment and advanced adenomas did not reach statistical significance (fixed-effects, RR = 0.92, 95%CI: 0.75-1.13; random-effects, RR = 0.92, 95%CI: 0.71-1.18; moderate quality of evidence).

CONCLUSION: Our results suggest a modest chemopreventive effect of calcium supplements against recurrent colorectal adenomas over a period of 36 to 60 mo. Further research is warranted.

Key words: Calcium; Colorectal adenoma; Recurrence; Cancer chemoprevention; Colorectal cancer; Systematic review; Meta-analysis; Polyp

Core tip: To assess the efficacy of calcium supplementation in reducing the recurrence of colorectal adenomas, we conducted a systematic review and meta-analysis of randomized, placebo-controlled trials. We found a modest protective effect of calcium in prevention of adenomas (fixed-effects, RR = 0.89, 95%CI: 0.82-0.96; random-effects, RR = 0.87, 95%CI: 0.77-0.98; high quality of evidence). On the other hand, the association between calcium treatment and advanced ("high-risk") colorectal adenomas was not statistically significant (fixed-effects, RR = 0.92, 95%CI: 0.75-1.13; random-effects, RR = 0.92, 95%CI: 0.71-1.18; moderate quality of evidence). Further targeted research is warranted.

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INTRODUCTION

Colorectal cancer is a tumour resulting from a complex interaction between inherited susceptibility and environmental factors, as demonstrated by genetic, experimental, and epidemiological studies[1-3]. It represents the third most common malignancy, and the fourth most common cause of cancer deaths globally, accounting for 1.35 million new cases and 0.7 million deaths annually[4].

The magnitude of the colorectal cancer problem, and the failure of advanced disease chemotherapy to effect significant reductions in the respective mortality rates, indicate that an intensive approach to the prevention of this disease is necessary. Accordingly, research on chemopreventive agents for colorectal cancer has received much attention over the last 30 years. Among several promising compounds (including vitamins A, C, and E, folate and other B vitamins, aspirin, sulindac and other non-aspirin non-steroidal anti-inflammatory drugs, statins, bisphosphonates, selenium, and fiber)[5-16], calcium has also been studied. It was proposed by Newmark *et al*[17] that calcium binds bile acids in the bowel lumen, inhibiting their well-known proliferative and carcinogenic effects. In addition, calcium has demonstrated a direct antiproliferative effect on cells, as well as promoting cellular differentiation and death (apoptosis)[18]. Evidence from epidemiologic studies also suggests that higher calcium intake may reduce the risk of colorectal cancer[19].

Most colorectal tumors develop from adenomas arising from the lining of the intestine. Progression - described as the adenoma-cancer (or polyp-cancer) sequence - is characterized by morphological and histological changes[20]. For instance, a small tubular adenoma acquires villoglandular characteristics as it grows. On the molecular level, the adenoma-cancer sequence reflects an accumulation of genomic defects. Generally, a single adenoma has a risk of progressing into neoplasia of 0.25% per year[21], depending on its size, location, histological type, and the presence of dysplasia.

The standard treatment for colorectal adenomas is endoscopic resection that interrupts the progression to invasive disease[22]. However, even after polypectomy, rates of adenoma recurrence may be up to 50% within 3 years of follow-up[23,24]. That is why research on colorectal cancer prevention has often focused on prevention of recurrent adenomas. Assuming that the effects of chemopreventive agents on adenomas reflect those on cancer, this endpoint provides a convenient surrogate for the study of colorectal cancer prevention[25,26].

Contrary to expectations, the recent randomized placebo-controlled trial published by Baron *et al*[27] showed that daily supplementation with 1200 mg of calcium did not significantly reduce the risk of colorectal adenomas over a time period of 3 to 5 years. In view of earlier promising clinical trial data[28,29], we sought to obtain a comprehensive snapshot of the existing evidence on the clinical efficacy of calcium supplementation for the prevention of colorectal adenomas. Therefore, we carried out an updated

systematic review and meta-analysis of randomized controlled trials (RCTs) published in the peer-reviewed literature.

MATERIALS AND METHODS

Data sources and search strategy

To identify the studies of interest, we systematically searched the PubMed and Scopus bibliographic databases from their inception to 15 December 2015 (date of final search). Search terms included: "calcium" combined with "adenoma" or "polyp". The search was limited to RCTs and human studies. No language restrictions were applied.

We also searched the Cochrane Library for any recently published systematic review on the subject, the WHO International Clinical Trials Registry Platform, and the *ClinicalTrials.gov* website, for completed but unpublished studies.

Two authors (Bonovas S and Lytras T) independently reviewed titles and abstracts to identify studies for inclusion. The full texts of the selected articles were carefully examined for eligibility, and their reference lists (as well as those of relevant systematic reviews[30-33]) were also investigated to identify any studies missed by the electronic database search.

Selection criteria

Studies were eligible for inclusion if they were randomized, placebo-controlled trials assessing supplemental calcium intake for the prevention of recurrent colorectal adenomas. All studies had to include follow-up evaluation (*i.e.*, endoscopy) to confirm the presence or absence of adenomas. If the results of a study were reported in multiple publications and/or at multiple time-points, we selected the most updated publication and extracted the data for the maximum follow-up time reported, as long as it remained a randomized trial and fully reported the outcomes of interest.

Studies were excluded if they were observational; did not report (or provided insufficient data to calculate) the outcomes of interest; or evaluated multi-interventional therapies, in which the effect of calcium treatment could not be separated out. We did not apply restrictions on eligibility according to dosage, or duration of calcium supplementation.

Types of outcomes and data extraction

We analyzed the following two outcomes: (1) recurrence of colorectal adenomas (at least one adenoma detected during follow-up colonoscopies); and (2) recurrence of advanced or "high-risk" adenomas (defined as those that have a diameter $\geqslant$ 10 mm, villous or tubulovillous features, or severe dysplasia).

Data extraction was independently undertaken by two authors (Bonovas S and Lytras T) using a predesigned form. The following information was extracted from each study: first author, journal and year of publication, study design and duration, number and characteristics of participants, intervention parameters, and number of subjects with the outcomes of interest reported for the intervention and control groups.

Disagreements were resolved *via* consensus, referring back to the original articles.

Assessment of risk of bias

We assessed the risk of bias (RoB) in included studies using the Cochrane Collaboration's tool[34], which addresses the following key-domains: sequence generation; allocation concealment; blinding; incomplete outcome data; selective outcome reporting; and other sources of bias, such as extreme baseline imbalances in prognostic factors, etc. These items were considered for RoB assessment and were classified as "adequate" (low RoB), "inadequate" (high RoB), or "unclear" (uncertain RoB).

Studies reporting adequate procedures in all domains were classified as "low RoB", studies with inadequate procedures in at least one domain were classified as "high RoB", and those with unclear procedures in one or more domains were classified as "uncertain RoB". Discrepancies among reviewers were discussed and agreement was reached by consensus.

Data synthesis and analysis

The risk ratio (RR) was used to measure treatment effects. Study-level RRs along with their 95%CI were calculated using intention-to-treat data for study participants who completed the follow-up evaluation (*i.e.*, follow-up colonoscopies).

Meta-analyses were performed twice, assuming a fixed-effects model (Mantel-Haenszel approach[35]) and a random-effects model (DerSimonian-Laird approach[36]). Under a fixed-effects model, we assume that the included studies share a common true effect, and the pooled effect is an estimate of the common effect size. Under a random-effects model, we assume that the true effects vary between the studies, and the pooled effect is a weighted average of the effects reported in the different studies. The random-effects model often leads to broader confidence intervals (*i.e.*, it is a more conservative approach)[37].

The between-study heterogeneity was evaluated using the Cochran's Q test[38], with a 0.10 level of significance, and the I-squared metric[39], which describes the percentage of variation across studies that is due to heterogeneity rather than chance. I-squared values of less than 25%, 25%-50%, or higher than 50% indicate low, moderate, or high heterogeneity, respectively[40].

Publication bias was not assessed, because the relevant statistical tests lack power when the number of included studies is limited[41].

To express the treatment effect in clinical terms, we calculated the number needed to treat (NNT) to prevent one adenoma recurrence using the Mantel-Haenszel

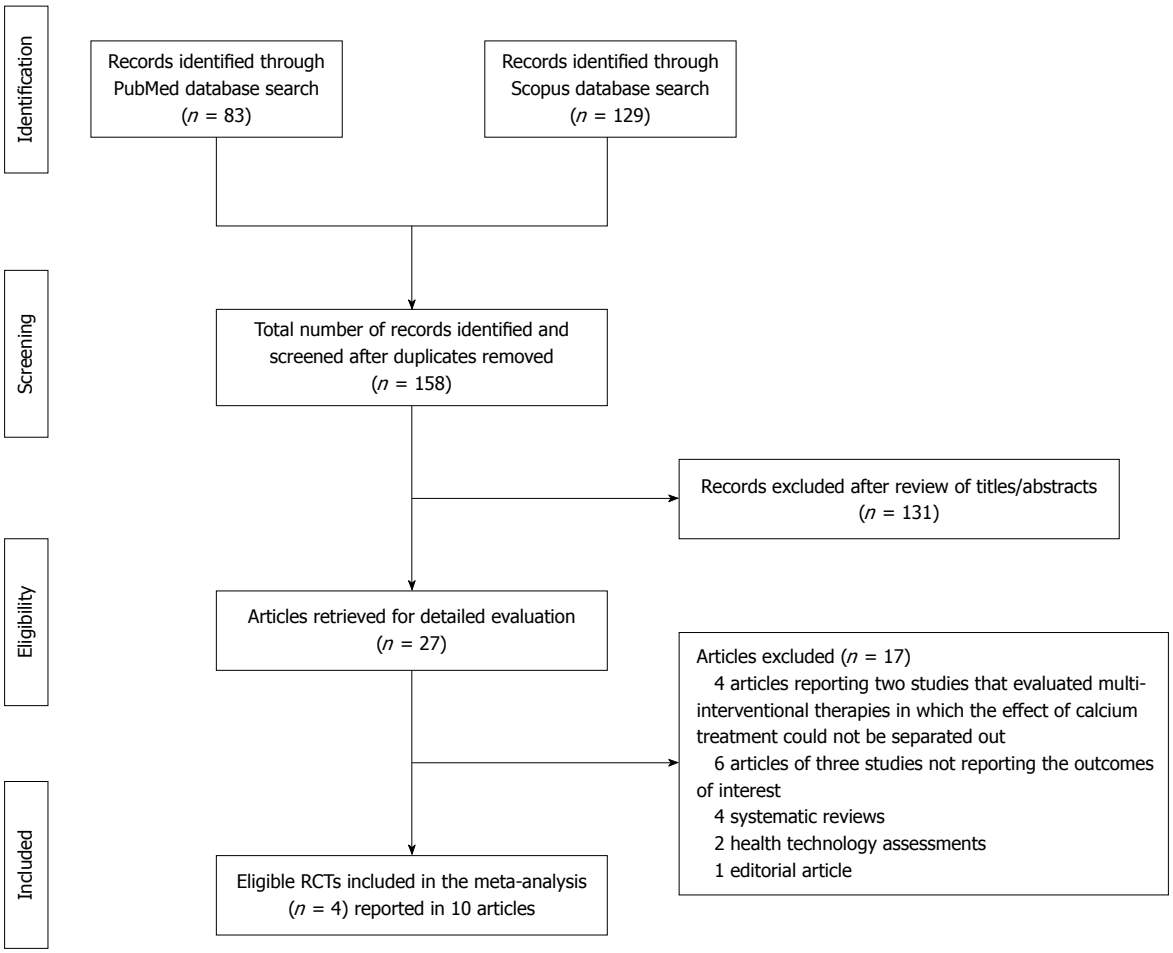

Figure 1 Summary of the evidence search and selection process (flow diagram). RCTs: Randomized controlled trials.

fixed-effects risk difference (risk in the placebo group minus risk in the calcium group), in cases in which a statistically significant RR was detected. The NNT is the inverse of this risk difference.

The quality of evidence (confidence in the synthesized effect estimates) was assessed using GRADE (Grading of Recommendations Assessment, Development and Evaluation)[42].

For all statistical analyses, we used the R software[43], version 3.2.2, and the "meta" package for R[44], version 4.3-0. All *P*-values are two-tailed. For all tests (except for heterogeneity), a *P*-value less than 0.05 indicates statistical significance.

Our study was performed in accordance with the Cochrane Handbook for intervention reviews[41], and the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-analyses) statement[45].

The study did not involve any experiment on humans or animals, thus an ethical approval was not required.

RESULTS

Search results

A summary of the literature search and selection process is shown in Figure 1 (Flow diagram). Four randomized studies of calcium supplementation met the eligibility criteria and were included: (1) the Vitamin D/Calcium Polyp Prevention Study[27,46]; (2) the Southwest Oncology Group (SWOG) Calcium Chemoprevention Pilot Study[47,48]; (3) the European Cancer Prevention Organisation (ECP) Calcium Fibre Polyp Prevention Study[28,49,50]; and (4) the Calcium Polyp Prevention Study[29,51,52].

They were multicenter, randomized, placebo-controlled trials of supplementation with calcium for the prevention of colorectal adenomas. Daily doses of elemental calcium ranged from 1200 to 2000 mg, while the duration of treatment and follow-up of patients ranged from 36 to 60 mo. A summary of the trials' characteristics is given in Table 1.

Patients underwent endoscopy at baseline; subsequent colonoscopies were then undertaken to assess adenoma recurrence during the follow-up. The studies differ in that the SWOG Calcium Chemoprevention Pilot Study[47,48] recruited patients with completely resected colorectal cancer, while all the other studies included participants with colorectal adenomas removed before enrollment. All studies reported the number of subjects with adenomas (and advanced adenomas) identified during the follow-up

Table 1 Randomized, double-blind, placebo-controlled trials of calcium supplementation for prevention of colorectal adenomas

Study or subgroup[1]	Participants randomized	Mean age (yr)	Women	Follow-up (mo)	Amount of elemental calcium supplemented (mg/d)
Vitamin D/Calcium Polyp Prevention Study[27]	1675	59	15%	36 or 60	1200
SWOG Calcium Chemoprevention Pilot Study[47]	220	68[2]	37%	60	1800
ECP Calcium Fibre Polyp Prevention Study[28]	439	59	37%	36	2000
Calcium Polyp Prevention Study[51,52]	930	61	28%	48	1200

[1]For the Vitamin D/Calcium Polyp Prevention Study, only a subgroup was taken into account in the analysis; [2]Median value. ECP: European Cancer Prevention Organisation; SWOG: Southwest Oncology Group.

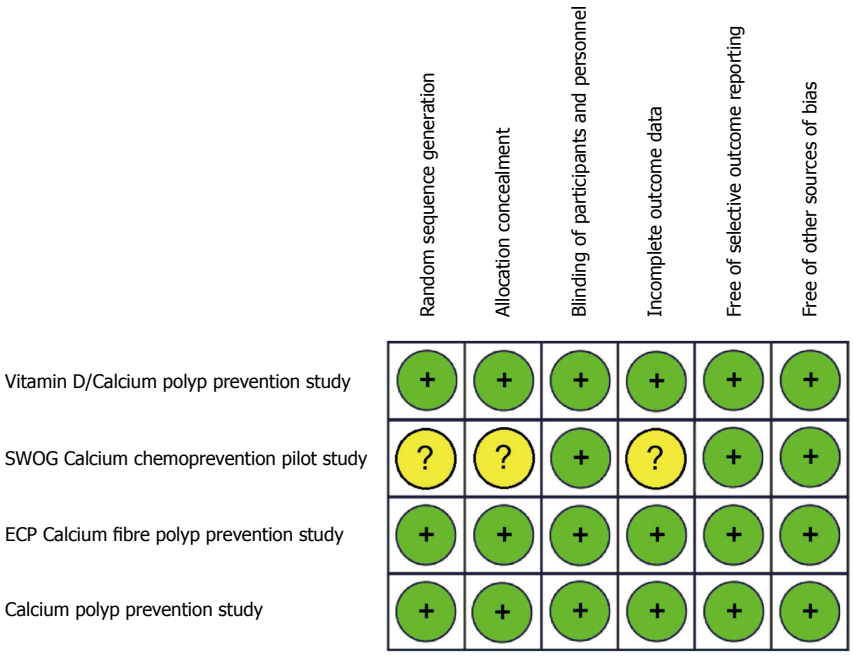

Figure 2 Risk-of-bias assessment for the studies included in the meta-analysis. Green (+): Low risk-of-bias; Yellow (?): Unclear risk-of-bias. ECP: European Cancer Prevention Organisation; SWOG: Southwest Oncology Group.

endoscopies; thus, we were able to conduct a post hoc analysis of these clinical trials, calculate RRs for the outcomes of interest, and incorporate them in the meta-analyses.

Another two clinical trials were identified, but did not meet the eligibility criteria, and were excluded. The first study - included in two previous systematic reviews[31,32] - examined a mixed intervention consisting of calcium, β-carotene, vitamin C, vitamin E, and selenium, compared with placebo[53,54]. The second clinical trial, which was evaluating a combination treatment of aspirin, calcitriol, and calcium, compared with placebo, was terminated early because no positive tendency was shown in a preplanned interim analysis[55,56]. In both studies, the effect of calcium could not be separated out; thus, they were not included in the evidence synthesis.

Risk of bias in included studies

The Vitamin D/Calcium Polyp Prevention Study[27,46], the ECP Calcium Fibre Polyp Prevention Study[28,49,50], and the Calcium Polyp Prevention Study[29,51,52], were judged to be at low RoB: their allocation sequences appeared to be adequately generated and concealed; patients and staff were masked; participants excluded from the analyses (those who had not undergone follow-up colonoscopy) were balanced in numbers and reasons across intervention groups; and the outcomes of interest for this review were fully reported.

The SWOG Calcium Chemoprevention Pilot Study[47,48] was considered to have uncertain RoB, because information was insufficient to permit judgement about the sequence generation process, the method used to conceal allocation, and attritions/exclusions.

Quality assessment items are presented in Figure 2.

Results of quantitative synthesis

Recurrence of colorectal adenomas: Intention-to-treat data for 2984 participants, who underwent follow-up colonoscopies, were analyzed. Each one of the four included trials reported a lower recurrence rate of colorectal adenomas in the calcium group, as compared to the placebo group; however, the results only from two studies (the Calcium Polyp Prevention

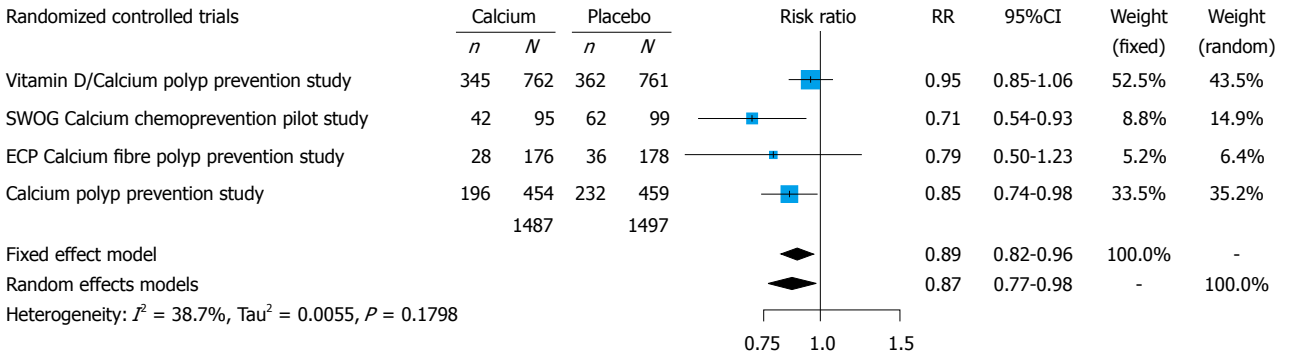

Figure 3 Forest plot for adenomas: results from individual studies and meta-analysis. For the analysis we used intention-to-treat data for patients who underwent follow-up evaluation (follow-up colonoscopies). ECP: European Cancer Prevention Organisation; SWOG: Southwest Oncology Group; *n*: Number of subjects with at least one adenoma detected during the follow-up evaluation; *N*: Number of subjects who underwent follow-up evaluation; RR: Risk ratio.

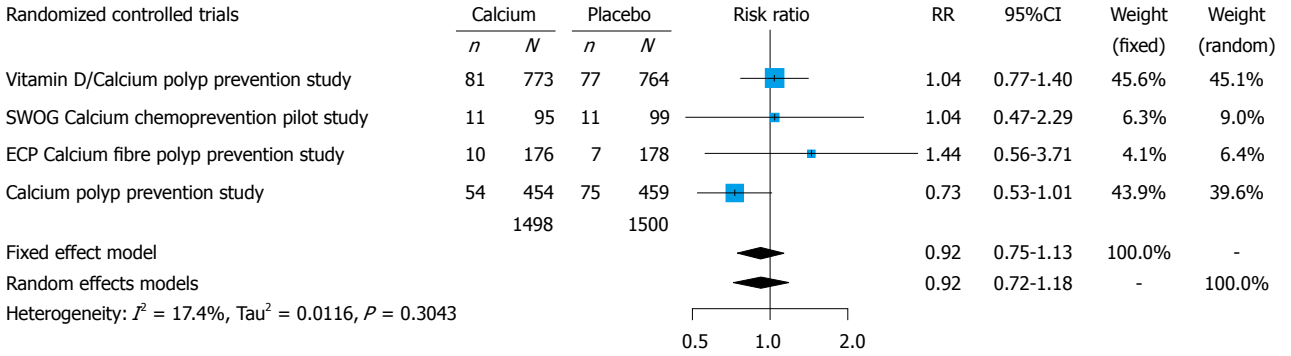

Figure 4 Forest plot for advanced adenomas: results from individual studies and meta-analysis. For the analysis we used intention-to-treat data for patients who underwent follow-up evaluation (follow-up colonoscopies). ECP: European Cancer Prevention Organisation; SWOG: Southwest Oncology Group; *n*: Number of subjects with at least one adenoma detected during the follow-up evaluation; *N*: Number of subjects who underwent follow-up evaluation; RR: Risk ratio.

Study[29,51,52] and the SWOG Calcium Chemoprevention Pilot Study[47,48]) were statistically significant. The overall recurrence rate, on all four RCTs, was 41.1% in calcium groups and 46.2% in placebo groups, over a treatment and follow-up period of 3 to 5 years.

We found a statistically significant modest protective effect (about 10%-15% risk reduction) of calcium supplements in the prevention of colorectal adenomas, both under the assumption of a fixed-effects model (RR = 0.89, 95%CI: 0.82-0.96) and a random-effects model (RR = 0.87, 95%CI: 0.77-0.98). The RRs with their 95%CIs for the individual studies, and the pooled results, are shown in Figure 3. The Cochran's *Q* test had a *P*-value of 0.18 and the corresponding I-squared value was 39%, indicating moderate heterogeneity between the studies.

For patients treated with calcium supplements in the included trials, the NNT was 20 (95%CI: 12-61) to prevent one colorectal adenoma recurrence within a period of 3 to 5 years.

Recurrence of advanced (high-risk) adenomas: We analyzed data for 2998 participants, who completed their follow-up evaluations (colonoscopies). None of the studies reported statistically significant results for advanced adenomas. Their overall occurrence, on all four RCTs, was 10.4% in calcium groups and 11.3% in placebo groups.

In meta-analysis, the association between calcium treatment and advanced adenomas did not reach statistical significance, either assuming a fixed-effects model (RR = 0.92, 95%CI: 0.75-1.13) or a random-effects model (RR = 0.92, 95%CI: 0.71-1.18). The RRs with their 95%CIs for the individual studies, and the pooled results, are presented in Figure 4. The Cochran's *Q* test had a *P*-value of 0.30 and the corresponding I-squared value was 17%, indicating low heterogeneity between the studies.

Quality of the evidence

Using the GRADE approach[42], our confidence in the synthesized evidence is "high" for the first outcome (adenomas), but "moderate" for the second one (advanced adenomas), for the following reasons: (1) the data were derived from RCTs, which are considered as the gold standard for assessing drugs[57]; (2) the synthesized effect estimates are precise for adenomas, but imprecise for advanced adenomas; (3) heterogeneity is low-to-moderate across studies; and (4) publication bias is not likely.

A high quality of evidence means that "we are very confident that the true effect lies close to that of the

Table 2 Summary of findings

	Illustrative comparative risks (95%CI)		
	Assumed risk Placebo	Corresponding risk Calcium	
Recurrence of adenomas (follow-up: 3 to 5 yr)	462 per 1000	411 per 1000 (379 to 444)	Relative effect (95%CI): RR = 0.89 (0.82-0.96) No. of patients with follow-up evaluation: 2984 No. of RCTs: 4 Quality of evidence (GRADE): ++++ (high)
Advanced adenomas (follow-up: 3 to 5 yr)	113 per 1000	104 per 1000 (85 to 128)	Relative effect (95%CI): RR = 0.92 (0.75-1.13) No. of patients with follow-up evaluation: 2998 No. of RCTs: 4 Quality of evidence (GRADE): +++- (moderate)

(1) the basis for calculating the assumed risk is the overall event rate across the trial groups receiving placebo; (2) the corresponding risk (calcium group) is based on the assumed risk and the relative effect estimate (risk ratio); (3) the relative effect estimate and its 95%CI come from a Mantel-Haenszel fixed-effects meta-analytic model; and (4) the overall quality of evidence is judged as "high" for recurrence of adenomas, and "moderate" for advanced adenomas. A high quality of evidence means that "we are very confident that the true effect lies close to that of the estimate of the effect", while a moderate quality of evidence means that "we are moderately confident in the effect estimate. The true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different"[58]. Population: Patients with colorectal adenomas removed before enrollment. Intervention: Calcium supplementation (1200-2000 mg/d) to prevent recurrence of adenomas. Comparison: Placebo. RR: Risk ratio; GRADE; Grading of Recommendations Assessment, Development and Evaluation; RCTs: Randomized controlled trials.

estimate of the effect", while a moderate quality of evidence means that "we are moderately confident in the effect estimate. The true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different"[58].

A summary of findings and strength of evidence is shown in Table 2.

DISCUSSION

Chemoprevention is a promising area of cancer research focusing on prevention of malignancies through pharmacological, biological, and nutritional interventions[59]. As first defined by Sporn[60], cancer chemoprevention uses natural, synthetic, or biologic agents to reverse, suppress, or prevent either the initial phase of carcinogenesis or the progression of malignant cells to cancer[61-65]. Regarding chemoprevention of colorectal cancer, several studies suggest that calcium may have chemopreventive potential[66-72]. Our knowledge on the underlying mechanism is incomplete. It has been proposed that calcium may protect against neoplasia in the large bowel by binding bile and fatty acids, thus decreasing their proliferative and carcinogenic effects on colonic epithelial cells[17].

Meta-analysis is a statistical methodology for combining the findings from independent studies[73]. We undertook this systematic review and meta-analysis to assess and synthesize the existing evidence on the efficacy of calcium supplements in prevention of colorectal adenomas.

In the recent literature, we have identified three systematic reviews with meta-analyses of RCTs examining the efficacy of calcium supplementation for the prevention of colorectal adenomas[31-33]. Carroll *et al*[31] and Shaukat *et al*[32] performed similar three-trial meta-analyses including the ECP Calcium Fibre Polyp Prevention Study[28,49,50], the Calcium Polyp Prevention Study[29,51,52], as well as the Hofstad *et al*[53,54] study that examined a mixed intervention consisting of calcium, β-carotene, vitamin C, vitamin E, and selenium, compared with placebo. Both meta-analyses found a significant 20% risk reduction associated with calcium. On the other hand, the third meta-analysis by Weingarten *et al*[33] reported a larger protective effect for calcium (OR = 0.74, 95%CI: 0.58-0.95) including only the ECP Calcium Fibre Polyp Prevention Study[28,49,50] and the Calcium Polyp Prevention Study[29,51,52], and excluding the Hofstad *et al*[53,54] study because of the use of antioxidants as a co-intervention. However, Weingarten *et al*[33] used the numbers of randomized subjects, rather than the numbers of subjects who completed the follow-up evaluation (colonoscopy), as the denominator in the analysis. This approach assumes that none of the subjects lost to follow-up experienced the outcomes[74,75]; however, this assumption does not appear to be valid.

In our study, a rigorous and extensive literature search was conducted; four eligible randomized trials were identified (the Vitamin D/Calcium Polyp Prevention Study[27,46], the SWOG Calcium Chemoprevention Pilot Study[47,48], the ECP Calcium Fibre Polyp Prevention Study[28,49,50], and the Calcium Polyp Prevention Study[29,51,52]); two further trials were excluded (the Hofstad *et al*[53,54] study and the Pommergaard *et al*[55] study[56]) because they evaluated multi-interventional treatments where the effect of calcium could not be separated out; data extraction was carefully undertaken by two independent investigators; and the evidence was synthesized using appropriate statistical techniques. Our results indicate a modest chemopreventive effect of calcium supplements against colorectal adenomas (approximately 10%-15% risk reduction; high quality

of evidence). However, this effect was not statistically significant for the advanced (high-risk) adenomas (imprecise pooled effect estimates; moderate quality of evidence). These findings extend the results of the primary trials and have important implications for future research.

The strengths of this systematic review should be weighed against a number of limitations. Firstly, the number of available studies was limited. Secondly, a colorectal adenoma typically requires 10-15 years to evolve into clinically invasive cancer[76]. Therefore, we did not examine whether calcium supplementation affects the progression of adenomas into invasive cancer. To address this question, studies with longer durations of treatment and follow-up are necessary. Thirdly, we could not analyze whether the dose of calcium affected the results; however, the dose range was relatively narrow in the included trials (range: 1200-2000 mg of elemental calcium daily).

Despite these limitations, our study is the most up-to-date meta-analysis on the topic and adheres to the recommended PRISMA reporting standards. Calcium does not appear to strongly reduce the risk of adenomas; however, there is high quality evidence suggesting a modest overall risk reduction, which might be a composite of an effect of calcium supplements in some populations (*e.g.*, the non-obese[27]) and some adenoma types (*e.g.*, the right-colon adenomas[28]), and lack of effect in others. Therefore, we consider that the recent negative results published by Baron et *al*[27] is not the end of the road for calcium as a potential chemopreventive agent against colorectal carcinoma; rather a new research approach is warranted. There is good reason to focus again on basic research, and perform clinical and epidemiologic studies to answer questions related to dosing and duration of treatment, and identify populations for whom calcium might be particularly beneficial for prevention of adenomas and colorectal cancer.

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COMMENTS

Background

Colorectal cancer is the 3rd most common malignancy and the 4th most common cause of cancer deaths globally, with 1.35 million new cases and 0.7 million deaths annually. Most colorectal malignancies develop from adenomas arising from the lining of the intestine. That is why research on colorectal cancer prevention has often focused on prevention of recurrent adenomas.

Research frontiers

It has been suggested that calcium binds bile acids in the bowel lumen, inhibiting their well-known proliferative and carcinogenic effects. Calcium has also demonstrated a direct antiproliferative effect on cells, as well as promoting cellular differentiation and apoptosis.

Innovations and breakthroughs

Contrary to expectations, the recent randomized controlled trial published by Baron *et al* showed that daily supplementation with 1200 mg of calcium did not significantly reduce the risk of colorectal adenomas over a period of 3 to 5 years. In view of earlier promising clinical data, the authors sought to obtain a comprehensive picture of the evidence by conducting a systematic review and meta-analysis of randomized controlled trials.

Applications

The results show a modest chemopreventive effect of calcium supplements against recurrent colorectal adenomas. Further clinical and epidemiological research is warranted to answer questions related to dosing and duration of treatment, and identify populations for whom calcium might be particularly beneficial.

Terminology

Meta-analysis is a statistical methodology for combining the findings from independent studies.

Peer-review

This study presented the effect of calcium supplementation on colorectal adenoma recurrence through a meta-analysis of clinical trials. This is a valuable paper on secondary adenoma prevention in the colon. The authors give a comprehensive view of an important clinical topic.

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CASE REPORT

Pseudo-Meigs' syndrome secondary to metachronous ovarian metastases from transverse colon cancer

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Author contributions: Kyo K served as an attending doctor for the patient, performed the surgery, and wrote the manuscript; Maema A, Shirakawa M, Nakamura T, Koda K and Yokoyama H were involved in editing the manuscript; and Koda K performed the pathological examination.

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Abstract

Pseudo-Meigs' syndrome associated with colorectal cancer is extremely rare. We report here a case of pseudo-Meigs' syndrome secondary to metachronous ovarian metastases from colon cancer. A 65-year-old female with a history of surgery for transverse colon cancer and peritoneal dissemination suffered from metachronous ovarian metastases during treatment with systemic chemotherapy. At first, neither ascites nor pleural effusion was observed, but she later complained of progressive abdominal distention and dyspnea caused by rapidly increasing ascites and pleural effusion and rapidly enlarging ovarian metastases. Abdominocenteses were repeated, and cytological examinations of the fluids were all negative for malignant cells. We suspected pseudo-Meigs' syndrome, and bilateral oophorectomies were performed after thorough informed consent. The patient's postoperative condition improved rapidly after surgery. We conclude that pseudo-Meigs' syndrome should be included in the differential diagnosis of massive or rapidly increasing ascites and pleural effusion associated with large or rapidly enlarging ovarian tumors.

Key words: Pseudo-Meigs' syndrome; Colon cancer; Ascites; Pleural effusion; Ovarian metastasis

Core tip: Pseudo-Meigs' syndrome associated with colorectal cancer is extremely rare. Here, we report a case of this syndrome secondary to metachronous

ovarian metastases from transverse colon cancer. This patient complained of progressive abdominal distention and dyspnea preoperatively, but her postoperative condition improved rapidly after bilateral oophorectomies. We conclude that pseudo-Meigs' syndrome should be included in the differential diagnosis of massive or rapidly increasing ascites and pleural effusion associated with large or rapidly enlarging ovarian tumors.

Kyo K, Maema A, Shirakawa M, Nakamura T, Koda K, Yokoyama H. Pseudo-Meigs' syndrome secondary to metachronous ovarian metastases from transverse colon cancer. *World J Gastroenterol* 2016; 22(18): 4604-4609 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i18/4604.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i18.4604

INTRODUCTION

Meigs' syndrome is defined by the presence of a benign ovarian tumor with ascites and pleural effusion that resolve after removal of the tumor[1]. The ovarian tumors in Meigs' syndrome are fibromas or fibroma-like tumors, and other benign or malignant pelvic tumors associated with ascites and pleural effusion are described as pseudo-Meigs' syndrome[2]. Pseudo-Meigs' syndrome associated with colorectal cancer is extremely rare, and the etiology of ascites and pleural effusion in this syndrome remains unknown. We describe here a case of pseudo-Meigs' syndrome secondary to metachronous ovarian metastases from transverse colon cancer and discuss the etiology of the ascites and pleural effusion in this syndrome.

CASE REPORT

A 65-year-old female with a history of surgery for ileus due to transverse colon cancer was admitted to our department for the induction of oxaliplatin-based chemotherapy. During the previous surgery, we had found numerous nodules of peritoneal dissemination, each 1 mm in size under the right diaphragm and in the pelvis, as well as sparsely distributed nodules of similar size on the mesentery of the small intestine. Furthermore, a disseminated nodule 5 mm in size had been observed in the greater omentum. We had performed right hemicolectomy with ileocolic anastomosis and omentectomy. Pathologically, moderately differentiated adenocarcinoma had been observed in the colonic tumor and in the resected nodule in the omentum, and no lymph node metastasis had been observed. After 1 year and 3 mo of treatment with irinotecan-based chemotherapy, computed tomography (CT) examination performed 22 d before admission revealed bilateral enlarged ovaries with solid and cystic components, suggesting bilateral ovarian metastases (Figure 1A). Although neither ascites nor pleural effusion had been observed in the CT examination, the patient had already gained more than 4 kg, and a moderate amount of ascites had been identified by an abdominal ultrasound examination performed 9 d before admission. Four days after admission, she complained of abdominal distention and bilateral edema of the legs, and an abdominal ultrasound examination completed on the same day identified massive ascites and a small amount of bilateral pleural effusion. She soon became unable to eat because of abdominal distention. She also complained of progressive dyspnea, and at worst, her oxygen saturation was 88% in room air. CT examination performed 26 d after admission showed a large amount of ascites and pleural effusion with left-side predominance (Figure 1B and C). Bilateral

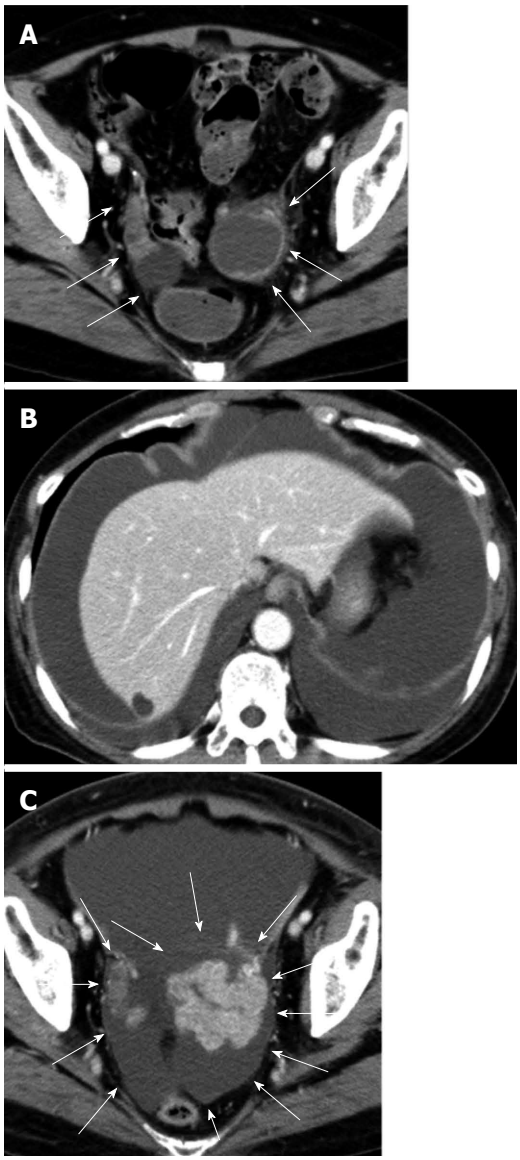

Figure 1 Computed tomography of the abdomen. A: The examination performed 22 d before admission showed bilateral enlarged ovaries with solid and cystic components (arrows). Ascites was not visible; B and C: The examination performed 26 d after admission revealed massive ascites and pleural effusion and rapid enlargement of ovarian tumors (arrows).

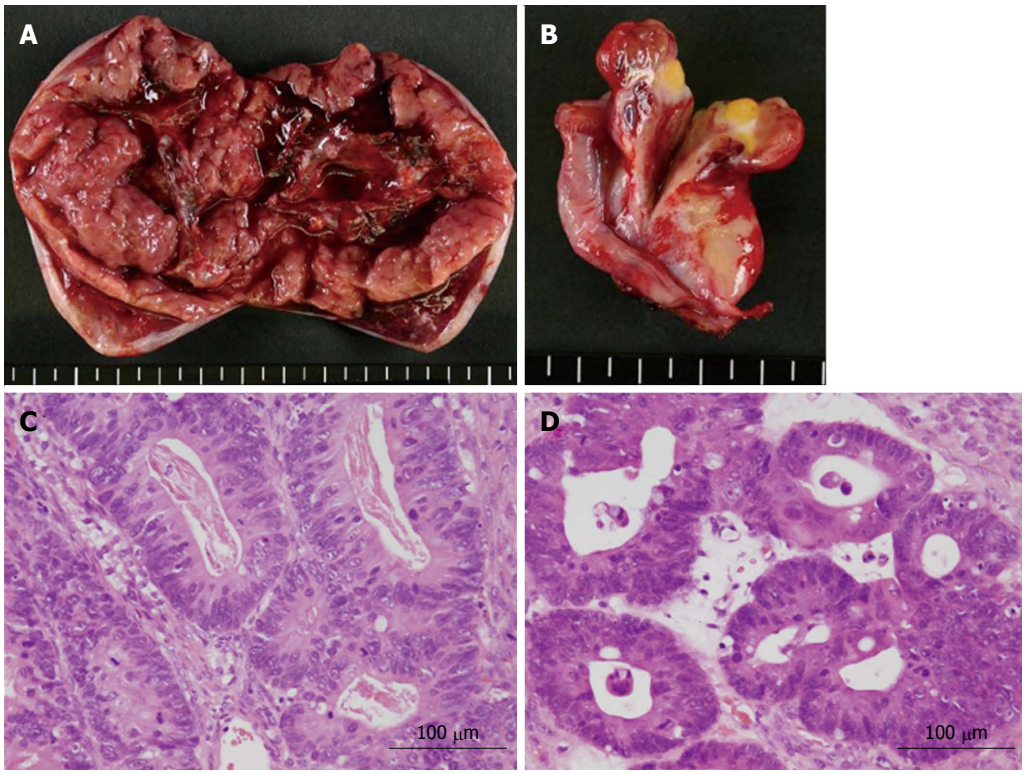

Figure 2 Pathological analysis. A, B: Cross-sectional views of the left and right ovarian tumors, respectively. The left ovarian tumor measured 90 mm × 55 mm, and the right ovarian tumor measured 30 mm × 30 mm. Both tumors contained solid and cystic components; C, D: Microscopic views of the left and right ovarian tumors, respectively, showing moderately differentiated adenocarcinoma.

ovarian tumors showed a rapid increase in size, and the left and right ovaries measured 97 mm × 66 mm and 80 mm × 29 mm, respectively (Figure 1B and C). Abdominocenteses were performed three times, and cytological examinations of the fluids were all negative for malignant cells. Despite the diffuse peritoneal dissemination observed during the previous surgery, we suspected pseudo-Meigs' syndrome caused by metastatic ovarian tumors and recommended the patient for surgery. Laboratory tests prior to surgery showed carcinoembryonic antigen (CEA) levels at 2.9 ng/mL (normal, < 5 ng/mL), carbohydrate antigen (CA) 19-9 levels at 51.4 U/mL (normal, < 37 U/mL), and CA 125 levels at 1150.0 U/mL (normal, < 35 U/mL). After thorough informed consent, a laparotomy was performed by lower abdominal incision, and more than 3.6 L of serous fluid was removed. Bilateral oophorectomies were performed, and a drainage tube was inserted into the Douglas' pouch. Although we could not inspect the right subphrenic region from the incision, we did not find any of the peritoneal nodules formerly observed on the mesentery of the small intestine and in the pelvis. The resected tumors from the left and right ovaries measured 90 mm × 55 mm and 30 mm × 30 mm, respectively (Figure 2A and B). Pathological examination revealed moderately differentiated adenocarcinoma of the ovaries that had negative immunohistochemical staining for cytokeratin (CK) 7 but positive staining for CK18, CK19 and CK20, leading to the diagnosis of bilateral ovarian metastases from colon cancer (Figure 2C and D). The patient's postoperative condition improved rapidly after surgery. The amount of fluid drained through the abdominal tube during postoperative day 1 was 150 mL, and the tube was removed 2 d after the surgery. Although the feeling of abdominal distention diminished soon after the surgery, both body weight and abdominal girth did not change for 3 d after the surgery but rapidly decreased thereafter (Figure 3). After hospital discharge, she was treated with chemotherapy, and a pulmonary metastasis was resected 1 year after the oophorectomies. Recurrence of peritoneal dissemination was suspected by CT examination 2 years and 4 mo after the oophorectomies, and jejuno-jejunal bypass was performed for jejunal obstruction 5 years and 2 mo after the oophorectomies. She died of cancer 6 years and 11 mo after the first operation and 5 years and 6 mo after the oophorectomies.

DISCUSSION

In 1937, Meigs and Cass[1] described 7 cases of ovarian fibromas associated with ascites and pleural effusion, and these manifestations were subsequently termed Meigs' syndrome[3]. Pseudo-Meigs' syndrome associated with colorectal cancers is extremely rare, and only 11 cases, including the present case, have been reported in the English literature (Table 1)[4-13]. Eight out of these 11 cases were reported in Japan, and most of the patients were under 60 years of age,

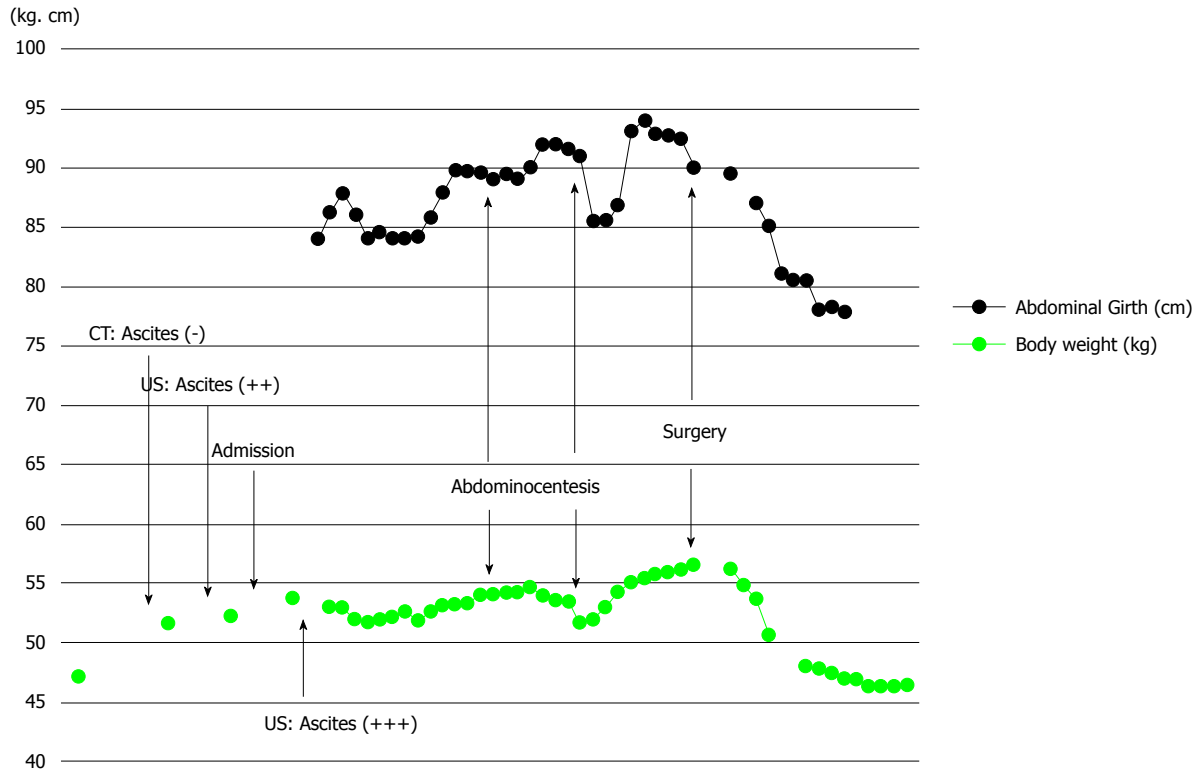

Figure 3 Line graphs showing changes in body weight and abdominal girth. Note that the body weight increased more than 4 kg before the emergence of ascites and that both the body weight and abdominal girth did not change for 3 d after surgery but thereafter decreased rapidly. CT: Computed tomography; US: Ultrasound.

Table 1 Characteristics of reported cases of pseudo-Meigs' syndrome associated with colorectal cancers

Ref.	Year	Age (yr)	Primary site	Onset of syndrome
Ryan[4]	1972	35	Transverse colon	Synchronous
Matsuzaki *et al*[5]	1992	39	Rectum	Synchronous
Nagakura *et al*[6]	2000	53	Sigmoid colon	Synchronous
Ohsawa *et al*[7]	2003	41	Sigmoid colon	Synchronous
Feldman *et al*[8]	2004	49	Cecum	Metachronous
Rubinstein *et al*[9]	2009	61	Cecum	Synchronous
Hosogi *et al*[10]	2009	44	Ascending colon	Synchronous
Okuchi *et al*[11]	2010	42	Rectum	Metachronous
Maeda *et al*[12]	2011	58	Sigmoid colon	Synchronous
Saito *et al*[13]	2012	44	Sigmoid colon	Synchronous
Present case	2016	65	Ascending colon	Metachronous

which is younger than the mean age for the development of colorectal cancers in the general population. There seems to be no preponderance in the locations of the primary lesions, and the associated ovarian metastases were metachronous in only 3 cases, including this case. Because of the metachronous development of this syndrome, we could observe the patient in detail from its early phase.

The etiology of ascites in this syndrome is unclear, although several theories have been proposed. First, Meigs suggested that irritation of the peritoneal surface by a hard solid ovarian tumor could stimulate the production of peritoneal fluid[14]. A second theory is that ascites develops due to pressure on the superficial lymphatics of the tumor[3]. A third theory is that stromal edema and transudation may occur as a result of a discrepancy between the arterial supply to a large tumor and the venous and lymphatic drainage of the same mass[15]. A fourth theory suggests that excessive production of fluid by the peritoneum leads to ascites in this syndrome[16]. The last but probably the most plausible theory is that increased capillary permeability and the resultant third-space fluid shift occur due to increased levels of inflammatory cytokines and vascular endothelial growth factor (VEGF)[17]. Abramov *et al*[17,18] reported high levels of interleukin (IL)-1beta, IL-6, IL-8, VEGF and fibroblast growth factor in serum, ascites and pleural effusion and a decline in serum levels of these inflammatory cytokines and vasoactive factors after removal of the ovarian tumor and suggested that the release of these factors from the tumor should be involved in the clinical manifestations of this syndrome by inducing capillary leakage and third-space fluid accumulation. The clinical manifestations of Meigs' syndrome show great similarity with a severe form of ovarian hyperstimulation syndrome (OHSS) that is associated with injection of human chorionic gonadotropin (hCG) and is also characterized by massive cystic enlargement of the ovaries, ascites and pleural effusion[19]. In severe OHSS, ascites and pleural effusion develop due to increased capillary permeability and resultant third-space fluid shift, and VEGF produced from ovarian follicles under the prolonged effects of hCG is thought to play a key

role[19,20]. The site of VEGF production in patients with pseudo-Meigs' syndrome is currently unclear. Okuchi *et al*[11] reported that immunohistochemical staining examinations showed increased VEGF expression in oviduct epithelial cells of both metastatic and non-metastatic sides rather than in tumor cells. Given that increased capillary permeability and resultant third-space fluid shift form part of the etiology of ascites in this syndrome, this may explain why the body weight of the present patient had increased well before the emergence of ascites and why both body weight and abdominal girth did not change for 3 d after removal of the ovarian tumors and insertion of a drainage tube.

The etiology of pleural effusion also remains unclear, but the prevailing theory is that the transfer of ascites occurs through transdiaphragmatic lymphatics, which was demonstrated by the flow of intraperitoneally injected India ink from the peritoneum to the pleural cavity[21]. Terada *et al*[22] also demonstrated that labeled albumin injected into the peritoneum was detected in the right pleura in maximum concentration within 3 h. However, considering the similarity of this syndrome to severe OHSS, increased capillary permeability may also be involved in the etiology of pleural effusion.

While it may be difficult, it is very important to distinguish the clinical manifestations of pseudo-Meigs' syndrome with those of disseminated malignant disease because in the case of this syndrome, removal of ovarian tumors will markedly improve not only the quality of life but also the prognosis of the patient. Thus, pseudo-Meigs' syndrome should be included in the differential diagnosis of massive or rapidly increasing ascites and pleural effusion associated with large or rapidly enlarging ovarian tumors.

COMMENTS

Case characteristics
The patient was a 65-year-old female who had surgery for ileus caused by transverse colon cancer.

Clinical diagnosis
Pseudo-Meigs' syndrome.

Differential diagnosis
Peritoneal carcinomatosis.

Laboratory diagnosis
Laboratory tests before oophorectomies showed carcinoembryonic antigen (CEA) levels at 2.9 ng/mL (normal, < 5 ng/mL), carbohydrate antigen (CA) 19-9 levels at 51.4 U/mL (normal, < 37 U/mL), and CA 125 levels at 1150.0 U/mL (normal, < 35 U/mL).

Imaging diagnosis
Bilateral ovarian metastases with massive ascites and pleural effusion.

Pathological diagnosis
Moderately differentiated adenocarcinoma in the ovaries, compatible with metastases from transverse colon cancer.

Treatment
Bilateral oophorectomies.

Related reports
Pseudo-Meigs' syndrome associated with colorectal cancer is extremely rare, and the etiology of ascites and pleural effusion in this syndrome remains unknown.

Term explanation
Meigs' syndrome is defined by the presence of a benign ovarian tumor with ascites and pleural effusion that resolve after removal of the tumor. The ovarian tumors in Meigs' syndrome are fibromas or fibroma-like tumors, and other benign or malignant pelvic tumors associated with ascites and pleural effusion are described as pseudo-Meigs' syndrome.

Experiences and lessons
This case report may provide supporting evidence for the etiology of ascites and pleural effusion in pseudo-Meigs' syndrome; our observations that the body weight of the patient had increased well before the emergence of ascites and that both the body weight and abdominal girth did not change for 3 d after removal of the ovarian tumors support the idea that increased capillary permeability and the resultant third-space fluid shift explain the etiology of ascites and pleural effusion in this syndrome.

Peer-review
The authors conclude that pseudo-Meigs' syndrome should be included in the differential diagnosis of massive or rapidly increasing ascites and pleural effusion associated with large or rapidly enlarging ovarian tumors.

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CASE REPORT

Isolated splenic metastasis from colon cancer: Case report

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Abstract

Isolated splenic metastases from colorectal cancer are very rare clinical entities and when they are present, they usually manifest widely disseminated disease. In this paper we report a case of metachronous solitary isolated splenic metastasis from colon cancer in a 64-year-old woman who was successfully treated by laparoscopic splenectomy. We discuss the pathological and clinical aspects of this condition. We furthermore comment on the diagnostic and therapeutic options of this rare entity through our observation of the case and consideration of the 31 case reports published in the literature.

Key words: Isolated splenic metastases; Colon cancer; Splenectomy

Core tip: Isolated splenic metastases from colon cancer are very rare, reporting of such cases was encouraged and recommended in several publications of this clinical entity to improve the management and survival of patients on stronger well-founded evidence.

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INTRODUCTION

Isolated Splenic metastases from colorectal cancer are very rare clinical entity and when they are present, they are usually a manifestation of widely disseminated disease[1]. This rareness can be explained by the anatomical, histological and functional characteristics of spleen which is usually considered an infertile soil for metastases[2]. We report a case of isolated splenic metastasis 16 mo after right Hemicolectomy for stage Ⅰ of colon adenocarcinoma which was successfully treated by Laparoscopic splenectomy. We discuss the clinical and pathological aspects of this rare entity, and consider the diagnostic and therapeutic options based on our observation of the case, and 31 cases reports published in the literature.

CASE REPORT

A healthy 64-year-old woman presented in July 2012, with abdominal pain and "heaviness". A colonoscopy was performed and demonstrated a budding tumor in the cecum that easily bleeds. A histological exam showed moderately differentiated adenocarcinoma. A computed tomography (CT) scan of the chest, abdomen and pelvis revealed a Wall-thickening of the cecum without others abnormalities. She underwent a right hemicolectomy with a histopathological finding of a moderately differentiated adenocarcinoma invading the muscularis propria. Twelve lymph nodes were removed and none showed metastases (pT2N0M0, Stage Ⅰ), with no blood vessel invasion. The status of MSI proteins and BRAF were not verified. she was on regular fallow-up every 3 mo with a serum carcinoembrionic antigen (CEA) and CT scan of the chest, abdomen and pelvis. Sixteen months after her colectomy, a serum CEA level was 38 ng/mL, CT Scan demonstrated a single low-density lesion in the spleen which measures 4.9 cm of diameter (Figure 1). At Laparoscopic exam, no intra-abdominal lesions and no peritoneum or liver metastases were detected. Splenectomy was performed at that time. The Histological exam demonstrated an adenocarcinoma with spread the splenic parenchyma (Figure 2). The following appointment, delayed at 6 mo post splenectomy, revealed no recurrences. CT scanning showed no recurrence in the spleen area, liver, abdomen or the chest; CEA level was 12 ng/mL, however a fluorodeoxyglucose-positron emission tomography (FDG-PET) scan revealed a high metabolic activity in the abdominal cavity, mediastinum and left inguinal lymph nodes suggesting diseases recurrences (Figure 3). Chemotherapy was therefore commenced consisting of Fluorouracil (5FU), Leucovorin, oxaliplatine and Bevacizumab. After 6 cycles, there was no evidence of progression disease.

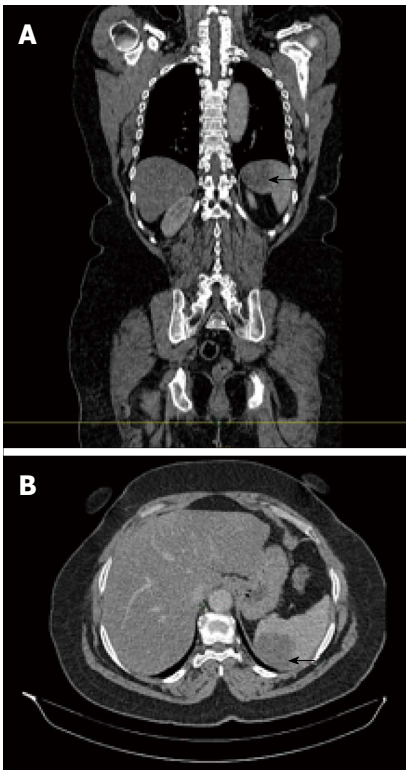

Figure 1 Abdominal computed scan demonstrating, in sagittal (A) and axial (B) views, a single low-density lesion of the spleen which measures 4.9 cm in diameter.

DISCUSSION

Splenic metastases from colorectal cancer are very rare. The incidence of this clinical entity was reported by Berge[2] to be 4.4% in 7165 autopsy cases, but he did not mention the incidence of Isolated Splenic metastases.

We found only 31 cases of isolated solitary Splenic metastasis from colorectal cancer in the English-language literature published in PubMed. dates searched were from 1969, date of the first case reported, to October 2015 (Table 1[3-31]). The cases consisted of 18 men and 13 Women, ranging in age from 33 to 84 years (mean, 62.7 years). The splenic metastasis was synchronous in five cases and metachronous in others 26 cases. The majority of cases were asymptomatic and the diagnosis was performed in follow-up of patients by imaging studies evaluation of increased CEA level which was elevated in 81% of cases ranging between 4.6 and 223 ng/mL, in our patient CEA level was 38 ng/mL. The interval between the primary tumor treatment and the detection of spleen metastasis varying from 3 to 144 mo (16 mo in our case). Splenic metastases were detected by Computed tomography scan, alone in 18 cases, or combined with other imaging studies such as Ultrosonography (5 cases), positron emission tomography (4 cases) and

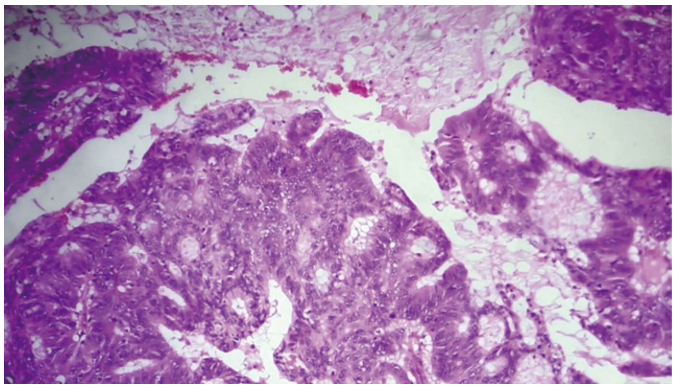

Figure 2 Adenocarcinoma spread the splenic parenchyma (hematoxylin-eosin staining, magnification × 20).

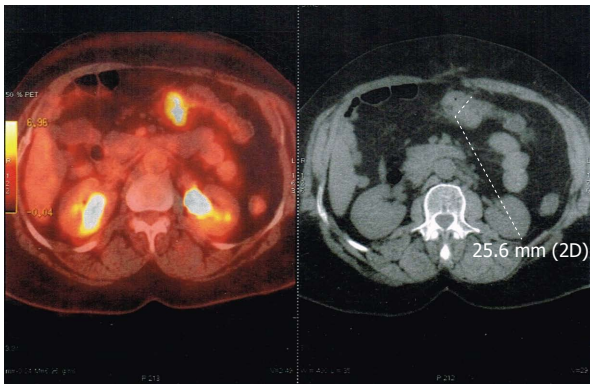

Figure 3 Fluorodeoxyglucose-positron emission tomography with high metabolic activity in the abdominal cavity.

Magnetic Resonance Imagery (1 case); 2 cases were detected by Liver-spleen scintigraphy; and 1 case by fluorodeoxyglucose-positron emission tomography (FDG-PET) scan[25]. The size of splenic lesions varied from 1.5 to 18 cm. In the cases reported the primary tumor was located in the sigmoid colon in 8 cases, in the ascending colon in 6 cases, in the descending colon in 5 cases, and in the rectum and Splenic flexure in 3 cases each; in the cecum in 2 cases as in our case, 1 case in the hepatic flexure and transverse colon each; In 2 patients the primary tumor localization was not specified. So the most frequent site of primary tumor is the left colon (61% of cases). Metastases are classically spread through either hematological or lymphatic pathways, however there is no lymphatic afferent in the spleen[1] and therefore the spread is *via* the blood vessels. in the cases of splenic metastasis, the regional lymph nodes were involved in 17 cases of primary tumor (16 cases with Sage Ⅲ, 1 case with Stage Ⅳ); in 9 cases the regional lymph nodes were negatives (7 cases with stage Ⅱ, 2 cases with stage Ⅳ), in 5 patients the regional lymph nodes was not specified; in our case the primary tumor was stage Ⅰ with no blood vessels invasion, it is the first in the literature. The diagnosis of spleen metastasis was confirmed by fine-needle aspiration in two cases[14,27] and in others cases by splenectomy.

Therapeutics options of metastatic colorectal cancer include surgery, chemotherapy with or without target therapy and radiotherapy. In all cases reported of Isolated Splenic metastases from colon cancer, splenectomy was performed, with Laparoscopic approach in only one case[25] as in our patient. This surgical technique remains controversial in Splenic malignancies because the risk of peritoneal dissemination and the few data in the literature. Lopez Monclova *et al*[31] reports 6 cases of Laparoscopic splenectomy for splenic metastasis from melanoma, ovarian cancer, colorectal cancer and malignant fibrous histiocytoma without surgical complications with a survival ranged from 2 mo to 11 years. Moreover, studies have demonstrated that laparoscopic techniques used in surgery of colorectal and gynecological cancer were not associated with a greater risk of intraperitoneal dissemination than conventional techniques[32]. Despite the fact that Chemotherapy is the most appropriate options of metastatic colon cancer by improving symptoms and survival, it was given in only 5 cases, as neoadjuvant in 2 cases and adjuvant in 3 cases. Target therapy was given only for 1 patient[23]. Interval survival after splenectomy ranging from 3 to 84 mo (mean, 22.5 mo). in patients with metachronous splenic metastasis only 2 cases were relapsed between 9 and 11 mo, in contrast 3 of 5 cases of synchronous splenic metastases were relapsed. Our patient was relapsed 6 mo post splenectomy, we suggest that this early relapse could be explained by lack of adjuvant chemotherapy post splenectomy rather than the surgical technique.

In conclusion, isolated splenic metastases from colorectal cancer are very rare clinical entity. A strict monitoring of patients with colon cancer after primary treatment should lead to early diagnosis of such metastasis. Splenectomy followed by adjuvant chemotherapy seems the optimal approach that can improve survival, despite small number of cases in the literature.

This is the first case in our institution and the first case of metachronous isolated splenic metastasis from colon cancer of which the primary tumor was stage Ⅰ.

Table 1 Characteristic of patients, their primary tumors, therapeutics and outcomes in the cases reports of isolated splenic metastasis from colorectal carcinoma

Case No.	Ref.	Age, sex	Primary tumor	Stage (LN)	Size (cm)	CEA (ng/mL)	Imaging	Treatment[1]	1st DFI (mo)	2nd DFI (mo)
1	Dunbar *et al*[3], 1969	F, 78	Rectum	Ⅲ(+)	18	64.0	CT	S	48	84
2	Waller *et al*[4], 1982	M, 72	Sigmoid	Ⅲ(+)	9	106.0	LSS	S	48	6
3	Slavin *et al*[5], 1986	M, 81	Cecum	Ⅲ(+)	NL	7.5	LSS	S	30	12
4	Capizzi *et al*[6], 1992	F, 51	Rectum	Ⅱ(-)	NL	13.5	CT	S	51	14
5	Thomas *et al*[7], 1993	F, 72	Descending	Ⅱ(-)	3	223.0	CT	S	144	12
6	Mainprize *et al*[8], 1997	F, 62	Descending	Ⅲ(+)	4	High	CT	S	42	12
7	Indudhara *et al*[9], 1997	M, 74	Sigmoid	Ⅱ(-)	9.5	23.4	CT	S	24	24
8-11	Ishida *et al*[10], 199	F, 73	Ascending (3 cases)	Ⅳ(NL)	1.5-7.5 (all cases)	NL	CT	S	Syn	72
		M, 62	Splenic flexure (1 case)	Ⅳ(NL)		NL	CT	S	Syn	24
		M, 52		NL		NL	US and CT	S	12	6
		M, 48		NL		NL	US and CT	S	24	3
12	Weathers *et al*[11], 1999	F, 33	Sigmoid	Ⅲ(+)	3.5	9.0	CT and MRI	S	3	12
13	Kim *et al*[12], 2000	M, 65	Ascending	Ⅲ(+)	5	14.9	CT	S	36	18
14	Lee *et al*[13], 2000	F, 60	NL	NL	NL	High	CT	S	108	5
15	Place[14], 2001	M, 51	Sigmoid	Ⅲ(+)	13	5.0	CT	S	72	6
16	Okuyama *et al*[15], 2001	M, 62	Sigmoid	Ⅲ(+)	3	N	US and CT	S	24	23
17	Avesani *et al*[16], 2001	F, 52	Descending	Ⅳ(-)	5	High	CT	S	Syn	12 relapse
18	Cavallaro *et al*[17], 2004	F, 55	Sigmoid	Ⅲ(+)	3	N	CT and PET	S	21	12
19	Hiraiwa *et al*[18], 2006	F, 49	Ascending	Ⅳ(+)	NL	36.7	CT	S	Syn	24 relapse
20	Avninder *et al*[19], 2006	M, 52	Sigmoid	ⅡA(-)	13	7.2	US and CT	Cmt, S	108	22
21	Gencosmanoglu *et al*[20], 2006	M, 76	Descending	Ⅲ(+)	6.5	High	CT and PET	S, Cmt	17	12
22	Pisanu *et al*[21], 2007	F, 54	Splenic flexure	Ⅳ(-)	4.5	High	CT	S, Cmt	Syn	6 relapse
23	Popović *et al*[22], 2008	M, 72	Rectum	Ⅲ(+)	NL	High	CT	S	18	NL
24	Bigot *et al*[24], 2008	F, 69	Sigmoid	Ⅱ(-)	4	High	CT	S	24	60
25	Gasent Blesa *et al*[25], 2008	F, 52	Descending	Ⅲ(+)	4.5	High	PET	S	36	NL
26	Montemurro *et al*[23], 2008	F, 80	Transverse	Ⅲ(+)	8	52.0	CT	S, TT	9	6
27	Sileri *et al*[26], 2009	M, 73	Hepatic flexure	Ⅱ(-)	1.5	High	CT-PET	S	60	40
28	Busić *et al*[27], 2010	M, 70	Splenic flexure	Ⅲ(+)	8	High	CT US	S	24	12
29	Genç *et al*[28], 2010	M, 59	Ascending	Ⅲ(+)	4	37.0	CT	S	15	24
30	Dogan *et al*[29],2010	M, 58	NL	Ⅱ(-)	3.5	4.62	CT	S, Cmt	20	11 relapse
31	Pavlović *et al*[30], 2011	M, 78	Cecum	Ⅲ(+)	7	38.6	CT, PET	Cmt, S	37	9 relapse
32	Our case, 2015	F, 64	Cecum	Ⅰ(-)	4.9	38.0	CT	S	16	6 relapse

[1]Spleen metastases; 1st DFI disease-free interval between treatment of primary tumor and diagnosis of the spleen metastasis; 2nd DFI disease- free interval after splenectomy. CEA: Carcinoembrionic antigen; N: Normal; LN: Lymph node; NL: Not listed; CT: Computed tomography; US: Ultrasonography; LSS: Liver splenic scintigraphy; PET: Positron emission tomography; MRI: Magnetic resonance imagery; S: Surgery; Cmt: Chemotherapy; TT: Target therapy.

We report this case for enrich the database of this rare clinical entity and to improve the management and survival of patients with isolated splenic metastases from colorectal cancer as was recommended and encouraged in similar publications.

COMMENTS

Case characteristics

A 64-years-old woman was on regular fallow-up post right hemicolectomy for stage I adenocarcinoma of the cecum. Computed tomography (CT) scan demonstrated a single low-density lesion in the spleen sixteen months post her hemicolectomy.

Clinical diagnosis

The patient was no symptoms; the spleen metastasis was detected after routine monitoring.

Differential diagnosis

Splenic infraction or abscess, lymphoma.

Laboratory diagnosis

A serum carcinoembrionic antigen level was 38 ng/mL.

Imaging diagnosis

CT scan demonstrated a single low-density lesion in the spleen which measures 4.9 cm of diameter.

Pathological diagnosis

An adenocarcinoma with spread the splenic parenchyma.

Treatment

Laparoscopic splenectomy.

Term explanation

Microsatellite instability (MSI) result in a defect of DNA mismatch repair, MSI/BRAF testing had a prognostic factor in colon cancer.

Experiences and lessons

A strict monitoring of patients with colon cancer after primary treatment should lead to early diagnosis of such metastasis and the properly treatment.

Peer-review

This paper is informative and is suitable for publishing.

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EDITORIAL

Current treatment of chronic hepatitis C in China: Dilemma and potential problems

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Abstract

Major advances have been made in the treatment of chronic hepatitis C virus (HCV) infection with the advent of direct-acting antiviral agents (DAAs). China has the most cases of HCV infection worldwide, but none of the DAAs has been approved in mainland China so far, and interferon (IFN)-α-based treatment remains the standard of care. HCV patients without response or with contraindications to IFN-based therapy have no alternative options. However, many patients buy DAAs, especially the generic forms of sofosbuvir, from other countries or areas. Under these circumstances, the use of these drugs may cause many predictable and unpredictable problems in ethics, law and medical practice. Given the obstacles of legal accessibility to DAAs and the potential problems of obtaining and using DAAs in China, the early launching of the DAAs in China or the legalization of buying drugs from areas outside China and using these drugs in China is an urgent issue and needs to be dealt with as soon as possible, in the interest of the patients.

Key words: Hepatitis C virus infection; Treatment; Direct-acting antiviral agent; Generics; China

Core tip: This article describes the current treatment situation of chronic hepatitis C virus infection in China and discusses the potential problems pertinent to the access and the use of direct-acting antiviral agents (DAAs), especially the use of generic DAAs from various sources.

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INTRODUCTION

Infection with hepatitis C virus (HCV) is a leading

cause of liver disease. Worldwide, an estimated 130-170 million people have HCV infection, and China has the most cases of HCV infection worldwide, with an estimated 29.8 million people[1]. A high proportion of people with HCV infection have developed advanced chronic liver diseases, including chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC).

The primary goal of treating chronic HCV infection is to achieve a sustained virologic response (SVR), which is defined as the absence of serum HCV RNA 12-24 wk after cessation of treatment. Patients achieving an SVR are considered cured in that 99% of patients who achieve an SVR remain undetectable for virus during long-term follow-up[2]. Achievement of SVR is associated with improved clinical outcomes. Pegylated interferon (Peg-IFN)-α 2a or 2b in combination with ribavirin (RBV) has been the standard of care for chronic HCV infection. However, treatment with Peg-IFN-α and RBV has limited efficacy. For instance, 48 wk of Peg-IFN and RBV therapy may achieve SVR in only 40% of patients with HCV genotype 1 infection[3]. Significant adverse events may accompany the duration of treatment[3-5], resulting in poor adherence and premature treatment discontinuation. Moreover, patients with decompensated liver disease, patients with HIV/HCV co-infection, patients who have comorbidity such as heart disease or chronic kidney diseases, patients who have had renal failure and renal transplantation, and patients who have undergone liver transplantation for HCV-associated liver disease may be contraindicated to or ineligible for the regimen of IFN and RBV. Patients who have a null or low response to the regimen of IFN and RBV and patients who are unwilling to take the drugs have no alternative effective treatments. Therefore, novel treatments that have more potent antiviral activity and fewer adverse effects and are eligible and compatible for patients with complex comorbidity in real life settings are urgently required.

Fortunately, major advances have been made in the treatment of chronic HCV infection, with the advent of direct-acting antiviral agents (DAAs) in recent years. Many regimens free of IFN or free of both IFN and RBV have been devised based on combination of new DAAs. These new regimens provide excellent efficacy with higher SVR rates and good safety profile with fewer side effects, and are of shorter duration of treatment. The patients also have a better treatment experience, higher adherence to treatment, and substantial improvement of health-related quality of life during treatment[6]. DAA combination regimens also provide high SVR rates in patients with various HCV genotypes, disease conditions and treatment experiences, including cirrhosis associated with HCV genotype 1[7,8], liver and kidney transplant recipients[9], HCV-genotype-1-infected patients with compensated cirrhosis who had not achieved SVR after successive treatments with Peg-IFN and protease-inhibitor regimens[10], and treatment-naïve and treatment-experienced patients co-infected with HIV and HCV genotypes 1-4[11].

CURRENT TREATMENT OF CHRONIC HCV INFECTION IN CHINA

Because of the unavailability of the novel DAAs, IFN-α or Peg-IFN-α in combination with RBV remains the current standard of care for chronic HCV infection in mainland China. Under these circumstances, HCV patients, especially some important and difficult to treat HCV patients, such as nonresponders to IFN and RBV treatment and those with relapse; patients with renal failure or heart disease; patients intolerant to the adverse events of and with contraindications to INF and RBV; patients with HCV-related cirrhosis and/or HCC; and patients with HIV/HCV co-infection have no other treatment options. In reality, however, the patients themselves are pragmatic. They try to seek help from other sources and find ways possible to obtain the drugs for the treatment of their disease. The high cost used to be one of the obstacles for some patients because of the unaffordability, but the cost is not an issue with the launching of generic drugs in some countries such as India. The price of the generic drugs is much lower than their brand-name counterparts. As a result, many HCV patients have bought or are going to buy DAAs, mainly sofosbuvir, a "blockbuster drug", from various regions or countries such as India and Bangladesh through different means, including through brokers, relatives and friends who have the opportunity to buy the drugs. The HCV patient population using DAAs, mainly sofosbuvir, from the above-mentioned sources is rapidly increasing in China.

DISCUSSION

Undoubtedly, most patients may benefit from the use of these drugs. Ethically and responsibly, doctors would be pleased to see that the patients have access to effective medicines and the probable cure of their disease. However, some problems may be encountered.

First, none of the DAAs including sofosbuvir, whether generics or brand name, has been approved by the China Food and Drug Administration. In this respect, the use of these drugs appears to be illegal in China. There is a similar but not identical example. Lu Yong, a Chinese man, who was diagnosed with chronic myeloid leukemia when he was aged 34 years in 2002, was arrested, jailed and then released by the police authorities in China early this year because of purchasing the Indian generic drug imatinib mesylate for himself and other patients. Although he appeared to be accused of selling "fake drugs", he knows nothing about the reasons for either being arrested or being released[12]. It is suggested that there are some legal issues and gaps.

Second, physicians may feel frustrated and embarrassed when they are consulted by patients with chronic HCV infection regarding the treatment of the disease. The doctors may tell the patients that there are many new drugs that are effective and have fewer adverse effects and may be suitable for their condition when they advise patients who are unsuitable for IFN and RVB, but none of the drugs is lawfully available in China. Of course, the doctors can let the patients wait for the availability of these drugs. However, some of the patients, such as those with decompensated liver disease and those awaiting organ transplantation, cannot wait because of the rapid progress of their disease and its life-threatening potential. Another situation is that the patients consult doctors about using drugs that they have bought from other countries, mostly from illegal sources and by illegal means, and the drugs may thus be regarded as "fake drugs". Additionally, the quality of the drugs may also not be guaranteed. In this situation, the doctors may place themselves at risk because they appear to guide their patients to use "fake drugs".

Third, because of the lack of other DAAs for rational combination, most of the patients take sofosbuvir in combination with RBV and some patients even use sofosbuvir alone for the treatment of their HCV infection with various treatment durations, irrespective of the HCV genotypes involved, the underlying liver disease (hepatitis or cirrhosis) and comorbidity. In reality, the situation may be rather more complex than expected. HCV genotype 1 infection accounts for most cases of HCV infection in China, but there are also other genotypes in China, including 2, 3 and 6[13].

Although the combination of sofosbuvir and RBV is a pan-genotypic regimen and may be applied for HCV genotypes 1-6, and this regimen remains the standard of care for genotypes 2 and 3, its efficacy was suboptimal in patients with HCV genotype 1, with an SVR of 54% for genotype 1b treatment-naïve hepatitis and 60% for genotype 1 treatment-naïve cirrhosis after 24 wk of treatment[14]. In the United States, the regimen of sofosbuvir and RBV for 24 wk in patients with genotype 1 infection is not recommended because of the longer treatment duration and lower expected SVR rates compared with other regimens[15]. The efficacy of this regimen for treatment-experienced genotypes 2 and 3 infection, with or without cirrhosis, was also suboptimal, with an SVR of 72% for genotype 2 treatment-experienced cirrhosis after 12 wk of treatment, an SVR of 77% for genotype 3 treatment-experienced hepatitis after 24 wk of treatment, and an SVR of 60% for genotype 3 treatment-experienced cirrhosis after 24 wk of treatment[14]. Based on these data, it is indicated that a proportion of patients with HCV genotype 1 or genotypes 2 and 3 treatment-experienced hepatitis or cirrhosis is using an non-optimal regimen for treatment. Therefore, it is anticipated that higher non-response and relapse rates may result from the use of this regimen.

Another concern is the possibility of treatment-emergent variants that may confer resistance to the antiviral treatment, although sofosbuvir has high genetic barriers to resistance[14]. Of note, a DAA used as monotherapy is not recommended because of the strong likelihood of treatment failure and the potential to induce resistance[15].

Moreover, the drug safety and drug-drug interactions pertinent to the use of sofosbuvir and RBV or sofosbuvir alone cannot be completely ignored. Sofosbuvir has a good safe profile and few drug-drug interactions. However, the safety of sofosbuvir in patients with some comorbidity, such as severe renal impairment (an estimated glomerular filtration rate < 30 mL/min/1.73 m^2) or end-stage renal disease on dialysis, is not well established, in that the levels of sofosbuvir and its metabolite are substantially elevated in such patients[15]. In contrast, co-administration of P-glycoprotein inhibitors, including anticonvulsants such as phenobarbital, antimycobacterials such as rifampin, ritonavir-boosted tipranavir, and St John's wort can decrease sofosbuvir concentrations[16]. Additionally, although sofosbuvir is not supposed to have significant pharmacological interactions with tacrolimus or cyclosporine[17], unexpected tacrolimus/cyclosporine reduction, which needs dosage adjustment, was observed in transplant recipients during sofosbuvir/RBV treatment for severe HCV infection recurrence. This raises the importance of awareness in the post-transplant HCV-recurrence setting when sofosbuvir is administered[18]. All these may place the patients with some comorbidity in unsafe conditions or bring new problems for the retreatment of patients if necessary.

CONCLUSION

As doctors, we hope for early launching of the DAAs, not only sofosbuvir but also other agents that can be rationally combined with sofosbuvir, in China, and the legalization of buying drugs including generic drugs from areas outside China and using these drugs in China, without further delay, in the interest of the patients. Presently, regulations concerning the usage of drugs, including generic drugs from other areas and international routes, should be established by the health authorities in China to meet the urgent needs. In the long run, China's health authorities need to reform fully their drug import policy and distribution system to satisfy legitimately and sensibly the therapeutic requirement of patients. In particular, the authorities need to guarantee the timely availability of novel drugs, even if they may be generics, for the urgent need of severely ill patients to improve survival and quality of life. At the same time, doctors may become confident to advise the use of such drugs without fear of violating the law or regulations concerning the quality of the drugs. We wish for the early arrival of a time when patients can easily and legally have access to effective drugs, and doctors can lawfully and

unimpeachably play their role in the treatment of HCV infection.

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TOPIC HIGHLIGHT

2016 Gastric Cancer: Global view

HER2 testing in gastric cancer: An update

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Abstract

Human epidermal growth factor receptor 2 (HER2) overexpression is increasingly recognized as a frequent molecular abnormality in gastric and gastroesophageal cancer. With the recent introduction of HER2 molecular targeted therapy for patients with advanced gastric cancer, determination of HER2 status is crucial in order to select patients who may benefit from this treatment. This paper provides an update on our knowledge of HER2 in gastric and gastroesophageal cancer, including the prognostic relevance of HER2, the key differences between HER2 protein expression interpretation in breast and gastric cancer, the detection methods and the immunohistochemistry scoring system.

Key words: Human epidermal growth factor receptor 2 testing; Gastric cancer; Immunohistochemistry; Scoring system; Trastuzumab

Core tip: It is clear that human epidermal growth factor receptor 2 (HER2) protein over-expression and gene amplification are much more heterogeneous in gastric cancer compared to breast cancer. Gastric and gastroesophageal tumors require a unique immunohistochemistry scoring system and interpretation expertise. We aimed to clarify the key differences in immunohistochemistry interpretation of gastric cancer, providing a practical update on HER2 testing and scoring.

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INTRODUCTION

Human epidermal growth factor receptor 2 (HER2), also known as CerbB-2 and ERBB2, is a proto-oncogene located on chromosome 17q21 that encodes a transmembrane protein with tyrosine kinase activity,

a member of the HER receptor family and is involved in signal transduction pathways, leading to cell growth and differentiation[1].

Amplification of the HER2 gene and overexpression of its product were first discovered in breast cancer and are significantly associated with worse outcomes[2]. Many studies have demonstrated that HER2 is also present in several other malignancies, including colorectal cancer, ovarian cancer, prostate cancer, lung cancer and, particularly, gastric and gastroesophageal cancer[3].

In gastric and gastroesophageal cancer, the frequency of HER2 overexpression varies widely in the literature; studies have yielded inconsistent findings regarding its prognostic relevance[4-12]. With the recent introduction of trastuzumab for the treatment of patients with advanced gastric cancer, the clinical demand for HER2 assessment is rapidly increasing. However, HER2 testing in gastric cancer differs from testing in breast cancer because of inherent differences in tumor biology, intratumoral heterogeneity of HER2 expression and incomplete membrane staining that are commonly observed in gastric tumors[13].

This paper aims to summarize the current evidence regarding HER2 in gastric and gastroesophageal cancer and to provide a practical update on HER2 testing and scoring that is essential for appropriate selection of patients who are eligible for treatment with trastuzumab.

RELEVANCE OF HER2 IN GASTRIC AND GASTROESOPHAGEAL CANCER

The frequency of HER2 overexpression in gastric and gastroesophageal cancer ranges from 4.4% to 53.4%, with a mean of 17.9%[4-14].

Although some small-scale studies have not demonstrated the prognostic properties of HER2[4,5,9,12], a larger number of studies indicate that HER2 is a negative prognostic factor, showing more aggressive biological behavior and higher frequencies of recurrence in HER2-positive tumors[1,6-8,11,14].

Given this controversy of HER2 prognostic values, a systematic review of a large number of studies was recently conducted in order to address this issue[14]. Forty-two publications with a total of 12749 patients were reviewed; the majority (71%) of the publications showed that a HER2-positive status was associated with decreased survival and clinicopathological features of tumor progression, such as serosal invasion, metastases and higher disease stage[14]. The results clearly set HER2 as a negative prognostic factor, suggesting that HER2 overexpression/amplification is a molecular abnormality that might be associated with the development of gastric cancer[7,14].

HER2 MOLECULAR TARGETED THERAPY

Trastuzumab is a monoclonal antibody directed against HER2; as one of the first molecular-targeted drugs to be developed, it was first introduced for the treatment of HER2-positive advanced breast cancer[2].

There is no consensus on the mechanism in which trastuzumab acts in cancer cells, but the evidence is that in addition to preventing dimerization of HER2 with other HER family members and stimulating endocytosis, it seems to induce cell mediated immunity and inhibit angiogenesis[15].

In the ToGA trial, patients with HER2-expressing unresectable gastric and gastroesophageal tumors were treated with chemotherapy and trastuzumab or with chemotherapy alone. A statistically significant increase in overall survival was observed in patients who received trastuzumab[16].

Although only a modest improvement of 2.7 mo in the median overall survival was observed in HER2-positive patients with the addition of trastuzumab, according to the ToGA trial, there was an improvement of 4.2 mo in the median overall survival in a post-hoc analysis[14,16-18].

Other molecular HER2-targeted agents have been tested or are currently being tested such as pertuzumab, lapatinib, the antibody-drug conjugate trastuzumab-emtansine (TDM-1)[19-23] and afatinib (NIH study trial registration number NCT01522768; ClinicalTrials.gov). However, the efficacy of these agents has been shown to be either unsatisfactory or as modest as trastuzumab[22,24]. Trastuzumab is the first molecular targeted agent approved as a standard treatment in gastric cancer, but it remains under investigation for more potent utilization.

Thus, it is imperative to determine the HER2 status in advanced gastric or gastroesophageal junction adenocarcinoma in order to select patients who may benefit from this promising treatment.

HER2 TESTING METHODS

HER2 status is mainly assessed by immunohistochemistry (IHC) or *in situ* hybridization (ISH) assays. Both methods can be done on formalin-fixed and paraffin-embedded biopsy tissues or surgical specimens and occasionally, cytological samples[25]. Fluorescent *in situ* hybridization (FISH) is regarded to be the gold standard; however, because of its higher cost and time consumption, as well as the need for a fluorescence microscope, generally only equivocal cases are subjected to this technique. Furthermore, the high concordance between FISH and IHC that is reported in the literature supports the use of IHC, the most familiar and readily accommodated method in most surgical pathology laboratories[26-29].

Thus, IHC should be used as the first screening method for HER2 evaluation and those cases with results considered equivocal for HER2 overexpression (2+) should be referred for FISH analysis or other alternative *in situ* hybridization method[28] (Figure 1). A simple and practical alternative to FISH for these

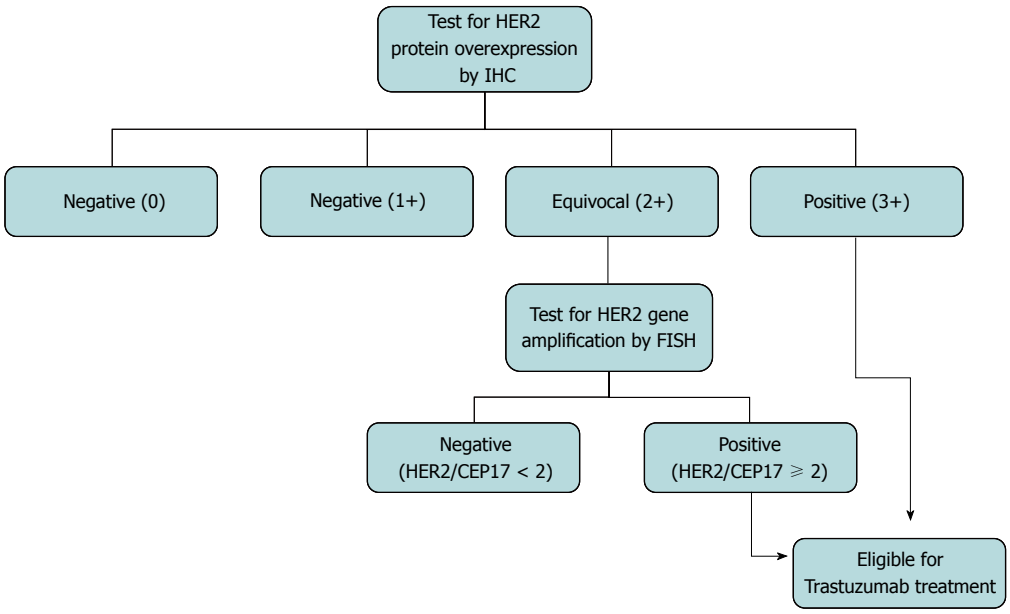

Figure 1 Human epidermal growth factor receptor 2 testing algorithm. HER2: Human epidermal growth factor receptor 2; IHC: Immunohistochemistry; FISH: Fluorescent *in situ* hybridization; CEP17: Chromosome 17.

Table 1 Advantages and disadvantages of the human epidermal growth factor receptor 2 testing methods

Method	Advantages	Disadvantages
IHC	Quick to perform; Most laboratories use fully automated processes; Widely used and familiar to all pathologists; Results can be viewed using a conventional bright-field microscope; Permits parallel viewing of tumor cell morphological features; Stained tissues do not degrade over time	Equivocal cases (2+) need another method for conclusion; Accuracy is more dependent on pre-analytic variables
FISH	Very objective and accurate; Actual copies of HER2 genes can be counted; Considered the golden standard of HER2 testing	Technically more demanding; Usually performed only in large laboratories/institutions; Costs are substantially high; Requires the use of fluorescence microscope and dark room; Comparatively more time consuming; Reagents degrade over time
SISH/CISH/ DDISH	Quick to perform; Very objective and accurate; Technique is fully automated; Results can be viewed using a conventional bright-field microscope; Permits parallel viewing of tumor cell morphological features; Slides can be stored because the signal is stable; Double-stranded probes labeled with two haptens can detect both markers on a single slide (DDISH)	More expensive than IHC; Unfamiliar to non-specialist pathologists

IHC: Immunohistochemistry; FISH: Fluorescent *in situ* hybridization; SISH: Silver *in situ* hybridization; CISH: Chromogenic *in situ* hybridization; DDISH: Dual-color dual-hapten *in situ* hybridization.

equivocal cases is provided by the employment of other *in situ* hybridization techniques such as silver *in situ* hybridization (SISH), chromogenic *in situ* hybridization and dual-color dual-hapten *in situ* hybridization. These three methods can be easily analyzed under a conventional bright field microscope and have shown excellent correlation with results obtained by FISH[30-32].

Because IHC is the easiest, least expensive and most widespread method of HER2 assessment, this paper focuses on IHC. Table 1 shows the different HER2 methods and their advantages and disadvantages.

Differences between HER2 expression in breast and gastric cancer

The key differences between HER2 expression in breast and gastric and gastroesophageal cancer are listed[17,30]: (1) the membranous distribution of the antibody in the neoplastic cells of breast cancer is

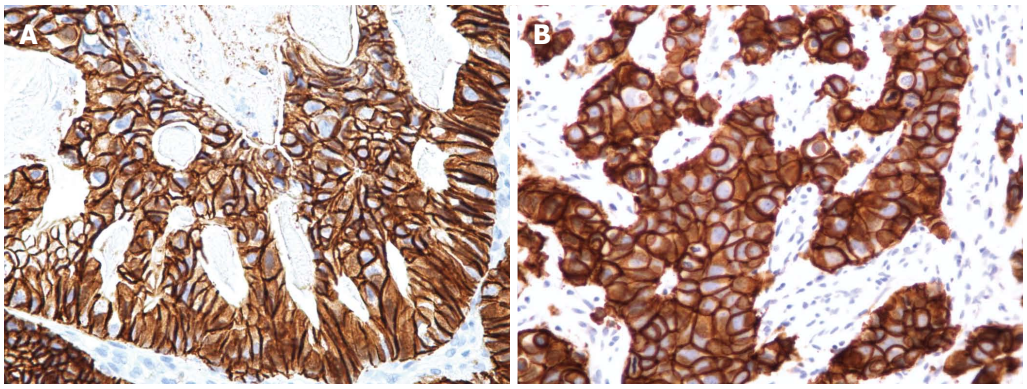

Figure 2 Human epidermal growth factor receptor 2 expression in gastric and breast tumors. A: A HER2-positive (3+) case of gastric adenocarcinoma; the cytoplasmic membranous immunostaining is incomplete and predominantly basolateral (× 400); B: A HER2-positive (3+) case of invasive ductal carcinoma of the breast; the cytoplasmic membranous staining is fully circumferential (× 400). HER2: Human epidermal growth factor receptor 2.

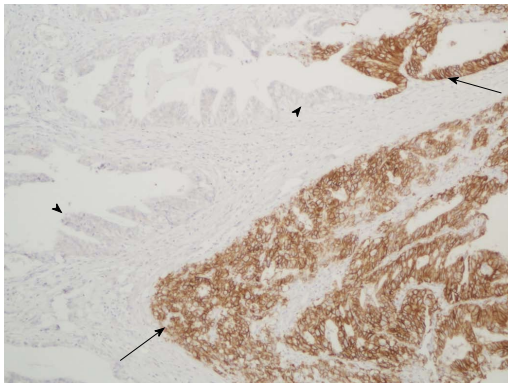

Figure 3 Representative image of the intratumoral heterogeneity of HER2 expression. Arrows indicate areas with strong continuous membranous staining (score 3+) and arrowheads indicate negative areas (score 0) (× 100). HER2: Human epidermal growth factor receptor 2.

predominantly circumferential, whereas in gastric cancer, it is generally incomplete, predominantly basolateral ("U"-shaped) or lateral (parallel lines) (Figure 2). Thus, unlike for breast cancer, circularity of IHC staining is not a criterion for HER2 IHC scoring in gastric cancer; (2) intratumoral heterogeneity, defined as the presence of areas with different HER2 scores within the same tumor, *i.e.*, focal or patchy positivity, is a common pattern encountered in gastric tumors but is only rarely seen in breast cancer (Figure 3). It may cause sampling errors when randomly sampled biopsies are examined (see below). Although the causes of intratumoral heterogeneity of HER2 expression are not yet fully understood, some studies indicate that it could be explained merely by tumor inherent genetic heterogeneity[33,34]. Since *Helicobacter pylori* (*H. pylori*) is widely accepted as the main causative agent of gastric cancer[35], we speculate whether among the diverse bacterial factors, concomitant infection with different strains and diverse host responses there could be a reasonable link with HER2 intratumoral heterogeneity. Interestingly, Tegtmeyer *et al*[36] showed that some *H. pylori* strains could in fact activate HER2, while infection with other strains suppressed HER2 activity. However, this correlation of the bacterium with HER2 intratumoral heterogeneity is still a matter of debate and requires further studies; and (3) variation of the incidence of HER2 expression with anatomic location does not occur in breast cancer, whereas it is more frequent in the proximal stomach, including the esophageal-gastric junction, than in the distal stomach. With the introduction of the seventh edition of TNM classification, a large number of tumors that were formerly categorized as gastric are now considered as esophageal and gastroesophageal junction tumors instead, with relatively high HER2-positivity rates in these primary neoplasms[37].

IHC score system

Given these differences between HER2 expression in breast and gastric cancer, an appropriate scoring system, exclusive for gastric tumors, was developed, because just transferring the breast cancer IHC scoring roles to gastric cancer could lead to a significant loss of patients. The system proposed by Hofmann *et al*[38] that has been assimilated by CAP and FDA, besides being specific for gastric tumors, also distinguishes biopsies from surgical specimens[17]. Table 2 shows the IHC score system for HER2 in gastric cancer and Figure 4 illustrates it.

Differences among samples

As mentioned above, mainly because of intratumoral heterogeneity, the size of the tissue sample might interfere in HER2 analysis. Although Hofmann's HER2 scoring system was formulated for evaluating HER2 status in biopsy and surgical specimens, discordant HER2 results in paired specimens were observed in a small percentage of tumors[39]. Intratumoral heterogeneity appears likewise to be the subject of conflicting results of HER2 expression in primary and metastatic tumor samples[33]. Moreover, in a previous study, we showed a significant difference in sensibility when analyzing HER2 expression in whole-tissue sections and in tissue microarrays[13]. Our personal

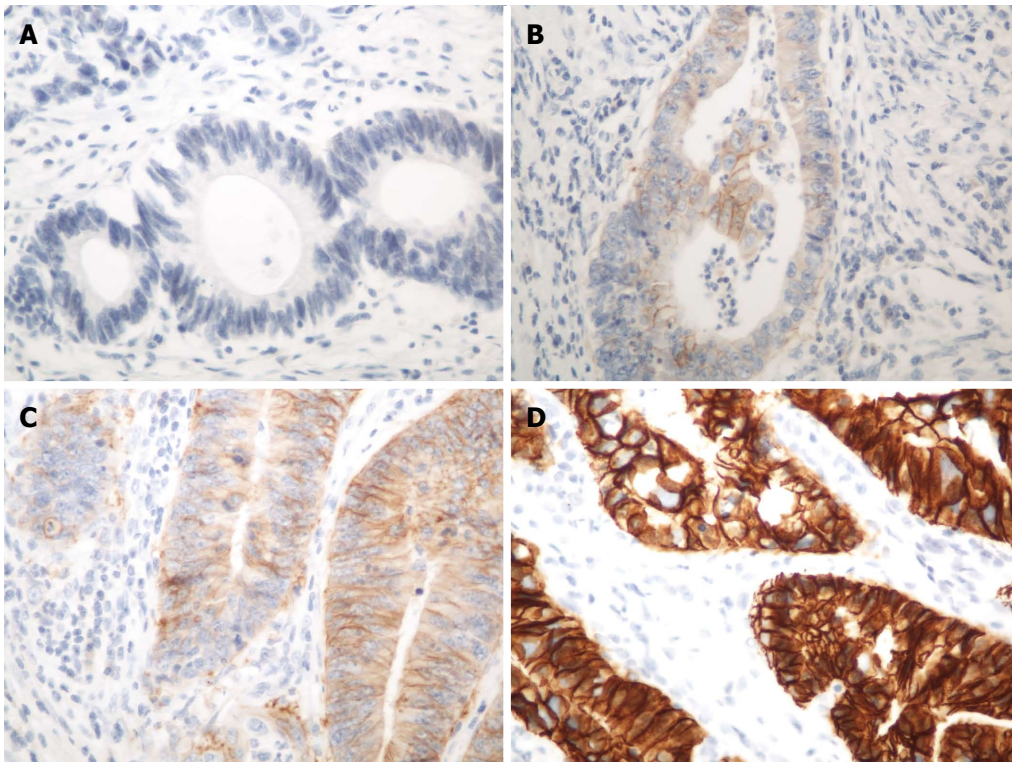

Figure 4 Human epidermal growth factor receptor 2 protein expression in gastric and gastroesophageal tumors. A: A negative (0) case; B: A negative (+1) case; C: An equivocal (2+) case; D: A positive (3+) case. HER2: Human epidermal growth factor receptor 2.

Table 2 Immunohistochemistry scoring for human epidermal growth factor receptor 2 expression in gastric and gastroesophageal junction cancer[17]

Score	Surgical specimen	Biopsy	HER2 overexpression assessment
0	No membranous staining or staining of < 10% of the tumor cells	No membranous staining or staining only in rare cells (less than 5 cohesive cells)	Negative
1+	Staining is weak or detected in only one part of the membrane in ⩾ 10% of the cells	Staining is weak or detected in only one part of the membrane of at least 5 cohesive cells	Negative
2+	Moderate/weak complete or basolateral membranous staining in ⩾ 10% of the cells	Moderate/weak complete or basolateral membranous staining of at least 5 cohesive cells	Equivocal
3+	Strong complete or basolateral membranous staining in ⩾ 10% of the neoplastic cells	Strong complete or basolateral membranous staining of at least 5 cohesive cells	Positive

HER2: Human epidermal growth factor receptor 2.

experience suggests that it is prudent to extend the evaluation to more than one sample and, if feasible, to also evaluate metastatic foci. In fact, testing all available specimens should be considered so that discrepancies can be excluded. When only biopsies are available, it is recommended to have at least four fragments containing tumor cells[40]. We also recommend that all surgical specimens from patients that previously obtained HER2-negative results in biopsies should also be tested to increase the chance of finding HER2-positive tumors.

IHC antibodies

The results of the HER2 test might differ according to the antibody used and, consequently, the antibody might considerably influence therapeutic decisions. An optimal IHC antibody should be adequately sensitive to select the greatest possible number of candidates for treatment and should have the lowest possible false-positive rate in order to avoid overtreatment.

The commercial antibodies currently available are the HercepTest and A0485 (Dako, Glostrup, Denmark), SP3 (Labvision; Thermo Fisher Scientific, Fremont, CA, United States), 4B5 (Ventana Medical Systems, Tucson, AZ, United States) and CB11 (Novocastra, Newcastle upon Tyne, England). Some studies have shown substantial divergence among the antibodies regarding the results of HER2 expression in gastric tumors[13,29,41]. Our previous study compared HercepTest, SP3 and 4B5. We observed that the 4B5 and SP3 antibodies showed similar good performance, with high NPV (negative predictive value) and AUC (area under the ROC curve) values that indicated higher accuracy compared to the HercepTest[13]. Based

on these results and on our personal experience, we believe that 4B5 and SP3 antibodies are more reasonable for first-line tests than the HercepTest in gastric tumors.

CONCLUSION

Given the recent introduction of trastuzumab for the treatment of patients with advanced gastric cancer, assessment of HER2 status is now mandatory for selecting patients eligible for this treatment. Although the development of automated platforms and image analysis should broaden the availability of *in situ* hybridization technologies, immunohistochemistry continues to play an essential role in HER2 status assessment. The overall reliability of HER2 evaluation by IHC, however, can be affected by diverse pre-analytical, analytical and post-analytical variables. Therefore, gastric and gastroesophageal cancer requires a unique scoring system, but above all, it requires expertise in interpretation.

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TOPIC HIGHLIGHT

2016 Gastric Cancer: Global view

Minimally invasive surgery for upper gastrointestinal cancer: Our experience and review of the literature

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Abstract

Minimally invasive surgery (MIS) for upper gastrointestinal (GI) cancer, characterized by minimal access, has been increasingly performed worldwide. It not only results in better cosmetic outcomes, but also reduces intraoperative blood loss and postoperative pain, leading to faster recovery; however, endoscopically enhanced anatomy and improved hemostasis *via* positive intracorporeal pressure generated by CO_2 insufflation have not contributed to reduction in early postoperative complications or improvement in long-term outcomes. Since 1995, we have been actively using MIS for operable patients with resectable upper GI cancer and have developed stable and robust methodology in conducting totally laparoscopic gastrectomy for advanced gastric cancer and prone thoracoscopic esophagectomy for esophageal cancer using novel technology including da Vinci Surgical System (DVSS). We have recently demonstrated that use of DVSS might reduce postoperative local complications including pancreatic fistula after gastrectomy and recurrent laryngeal nerve palsy after esophagectomy. In this article, we present the current status and future perspectives on MIS for gastric and esophageal cancer based on our experience and a review of the literature.

Key words: Stomach neoplasms; Esophageal neoplasms; Minimally invasive surgical procedures; Postoperative complications; Robotic surgical procedures

Core tip: Minimally invasive surgery (MIS) for upper gastrointestinal cancer reduces intraoperative blood loss and postoperative pain, leading to faster recovery. It also results in better cosmetic outcomes. The impact of MIS on postoperative complications and long-term outcomes has been under debate. We have recently demonstrated that use of da Vinci Surgical System might reduce postoperative local complications including pancreatic fistula after gastrectomy and recurrent laryngeal nerve palsy after esophagectomy.

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INTRODUCTION

Gastric cancer (GC) is the fifth most common malignancy and the third leading cause of cancer death in the world in 2012[1]. Surgical resection remains the only curative treatment option, and regional lymphadenectomy is recommended as part of radical gastrectomy[2]. According to the Japanese Gastric Cancer Association (JGCA) Gastric Cancer Treatment Guidelines, D2 gastrectomy is recommended for advanced gastric cancer (AGC)[3,4]; however, D2 lymphadenectomy, especially when combined with splenectomy or pancreaticosplenectomy, has been reported to increase morbidity and mortality[5-8].

Esophageal cancer (EC) is the eighth most common malignancy and the sixth leading cause of cancer death in the world in 2012[1]. Similar to GC, surgical resection remains the primary curative treatment option, and regional lymphadenectomy is recommended as part of radical esophagectomy[9-12]. Esophagectomy, which requires thoracolaparotomic manipulation, is one of the most invasive operations in gastrointestinal (GI) surgery, being associated with significant morbidity and mortality[13,14].

Minimally invasive surgery (MIS), which was launched in the late 80's[15], has been characterized by minimal access using laparoscope or thoracoscope with CO_2 insufflation[16]. Although the impact of MIS on postoperative inflammatory response has still been unclear, it has been increasingly used for upper GI malignancies in an attempt to improve postoperative outcomes[13,17,18].

This article provides the updates on laparoscopic gastrectomy (LG) for GC and video-assisted thoracoscopic surgery esophagectomy (VATS-E) for EC, particularly focusing on our twenty-year experience in this field along with a review of previously reported and ongoing large prospective studies.

GASTRIC CANCER

LG for early gastric cancer

Since the first report of LG by Kitano *et al*[19] in 1994, LG for GC has gained popularity because of its beneficial short-term effects leading to improved quality of life (QoL) in comparison with open gastrectomy (OG), although many controversies still exist due to the lack of solid evidence on its long-term outcomes[20-24]. Therefore, LG had long been recognized as an investigational treatment even for early gastric cancer (EGC) but not as a standard procedure in Japan[25]. However, based on the results of the following multicenter phase Ⅱ trial conducted by the Japanese Clinical Oncology Group (JCOG) (JCOG0703)[26], the new Japanese Gastric Cancer Treatment Guidelines (ver. 4, issued in 2014) has turned to allow laparoscopic distal gastrectomy (LDG) for clinical stage Ⅰ disease as a standard treatment option[4].

JCOG0703

JCOG0703[26] was conducted to assess the safety of LDG with D1+ lymph node (LN) dissection for clinical stage Ⅰ GC. In this well-designed phase Ⅱ study, to control for the quality of surgery, only surgeons who had performed 30 or more LDGs and 30 or more open distal gastrectomies (ODGs) participated. A central review of the surgical procedure in all the patients was conducted by evaluating photographs taken during the procedure. Between 2007 and 2008, 176 eligible patients from 14 hospitals were enrolled. The incidence of anastomotic leakage or pancreatic fistula was primarily determined, resulting in only 1.7 % (3/173), which was much lower than the pre-specified threshold of 8%. Moreover, morbidity (Common Terminology Criteria for Adverse Events, CTCAE v3.0 Grade 3 or 4)[27] was 5.1%. Thus, the safety of LDG for clinical stage IA/IB disease was securely confirmed.

JCOG0912

On the basis of JCOG0703, a multicenter phase Ⅲ RCT of LDG *vs* ODG with D1+ nodal dissection for clinical stage Ⅰ GC (JCOG0912) has currently been conducted to determine the non-inferiority of LDG to ODG in terms of overall survival[28,29]. For quality control of surgery, surgeons were required to have experience with at least 30 LDGs as well as certification (or its equivalent) from the Japan Society for Endoscopic Surgery (JSES) according to the Endoscopic Surgical Skill Qualification System[30]. Between 2010 and 2013, 921 patients (LDG 462, ODG 459) were enrolled from 33 institutions. Regarding short-term outcomes of this study, LDG significantly improved blood loss, postoperative pain and recovery of bowel movement irrespective of extended operative time. There were no grade 3 or 4 (CTCAE v4.0[31]) intraoperative adverse events in either arm. No difference was observed in the overall proportion of in-hospital, non-hematological

Table 1 Ongoing randomised controlled trials on laparoscopic distal gastrectomy for advanced gastric cancer

	JLSSG0901	KLASS02	CLASS01
Country	Japan	Korea	China
Start year	2010	2011	2012
Phase	Ⅱ/Ⅲ	Ⅲ	Ⅲ
Intervention	LDG *vs* ODG	LDG *vs* ODG	LDG *vs* ODG
Inclusion criteria	cT2-4a cN0-2 (except bulky N2)	cT2-4a cN0/1	cT2-4a cN0-3 (except bulky LN)
Sample size	Ⅱ:180, Ⅲ:500	1050	1056
Primary endpoint	Ⅱ: morbidity Ⅲ: 3-year RFS	3-year RFS	3-year RFS

JLSSG: Japanese Laparoscopic Surgery Study Group; KLASS: Korean Laparoscopic Gastrointestinal Surgery Study; CLASS: Chinese Laparoscopic Gastrointestinal Surgery Study; LDG: Laparoscopic distal gastrectomy; ODG: Open distal gastrectomy; LN: Lymph node; RFS: Relapse-free survival.

grade 3 or 4 adverse events excluding biochemical data (LDG *vs* ODG, 3.3% *vs* 3.7%). The proportion of grade 3 or 4 serum AST/ALT increased was higher in LDG than ODG (16.4% *vs* 5.3%, $P < 0.001$). Thus, this trial has so far demonstrated that LDG performed by the credentialed surgeons was at least as safe as ODG in terms of adverse event and short-term clinical outcomes. The primary analysis of the long-term outcomes including overall survival and relapse-free survival is planned in 2018[29].

KLASS01

The Korean Laparoscopic Gastrointestinal Surgery Study (KLASS) group 01 trial is another multicenter (13 institutions) RCT to confirm oncological safety of LDG for EGC in comparison with ODG[32,33]. The primary endpoint of this study is 5-year overall survival. Surgeons had to have performed at least 50 cases of both LDG and ODG, and their institution should have performed more than 80 cases of both LDG and ODG, respectively. Between 2006 and 2010, 1416 patients (705 LDGs and 711 ODGs) were enrolled. Regarding short-term outcomes, LDG improved the overall complication rate (LDG *vs* ODG, 13.0% *vs* 19.9%, $P = 0.001$), particularly wound complication (LDG *vs* ODG, 3.1% *vs* 7.7%, $P < 0.001$). The major intra-abdominal complication (LDG *vs* ODG, 7.6% *vs* 10.3%, $P = 0.095$) and mortality rates (LDG *vs* ODG, 0.6% *vs* 0.3%, $P = 0.687$) were similar between the groups. Thus, this trial has so far demonstrated that LDG for patients with clinical stage Ⅰ GC was sufficiently safe and has a benefit of lower occurrence of wound complication compared with conventional ODG. The long-term outcomes are being awaited.

Laparoscopic total gastrectomy for EGC

These multicenter prospective studies only cover distal gastrectomy. At this moment, both JGCA and JSES have commented that Laparoscopic total gastrectomy (LTG) should be cautiously introduced because of its technical difficulties in complicated alimentary tract reconstruction as well as the LN dissection at the splenic hilum or along the short gastric arteries[4,34]. Since techniques for laparoscopic esophagojejunostomy has recently been established among expert laparoscopic surgeons[35,36], JCOG is planning a phase Ⅱ study to determine the safety of LTG with D1+ LN dissection for clinical stage Ⅰ disease[37]. KLASS group has already been conducting a similar phase Ⅱ trial (KLASS03) to properly evaluate the perioperative morbidity and mortality of LTG for EGC since 2012[17].

LG for AGC

Application of LG for AGC remains to be debated not only because of the lack of evidence on long-term outcomes, but also because of the technical difficulty in performing complete D2 LN dissection and a concern for the innate risk of cancer cell dissemination to the peritoneal cavity[5,16,17,38]. Having said that, acceptable short- and long-term outcomes of the LG for AGC have been reported by a couple of experienced surgeons including us[38-41]. Currently, large-scale multicenter RCTs have been conducted in Japan (The Japanese Laparoscopic Surgery Study Group, JLSSG 0901[42]), Korea (KLASS02[17,43]), and China (The Chinese Laparoscopic Gastrointestinal Surgery Study, CLASS 01[17]) in order to determine the feasibility of LDG for locally AGC (Table 1).

LG for AGC at our institute

History: Laparoscopic surgery was launched in the early 90's in our country[44]. At that time, most laparoscopic surgeons applied laparoscopic surgery, using its minimally invasive nature, to less extended surgery[45]. However, we assumed from the beginning that laparoscopic surgery should be suitable for meticulous LN dissection using laparoscopically enhanced anatomy and reduced venous bleeding *via* pneumoperitoneum irrespective of the limited range of motion, poor depth perception, and limited tactile sensation[5,46]. Then, we introduced laparoscopic assistance into moderate to advanced GI surgery in combination with a caudocranial and mediolateral approach to overcome those limitations in 1995, and developed techniques for LDG and LTG with D2 dissection for AGC, which were published for the first time in the world[47,48]. Since then, we have performed

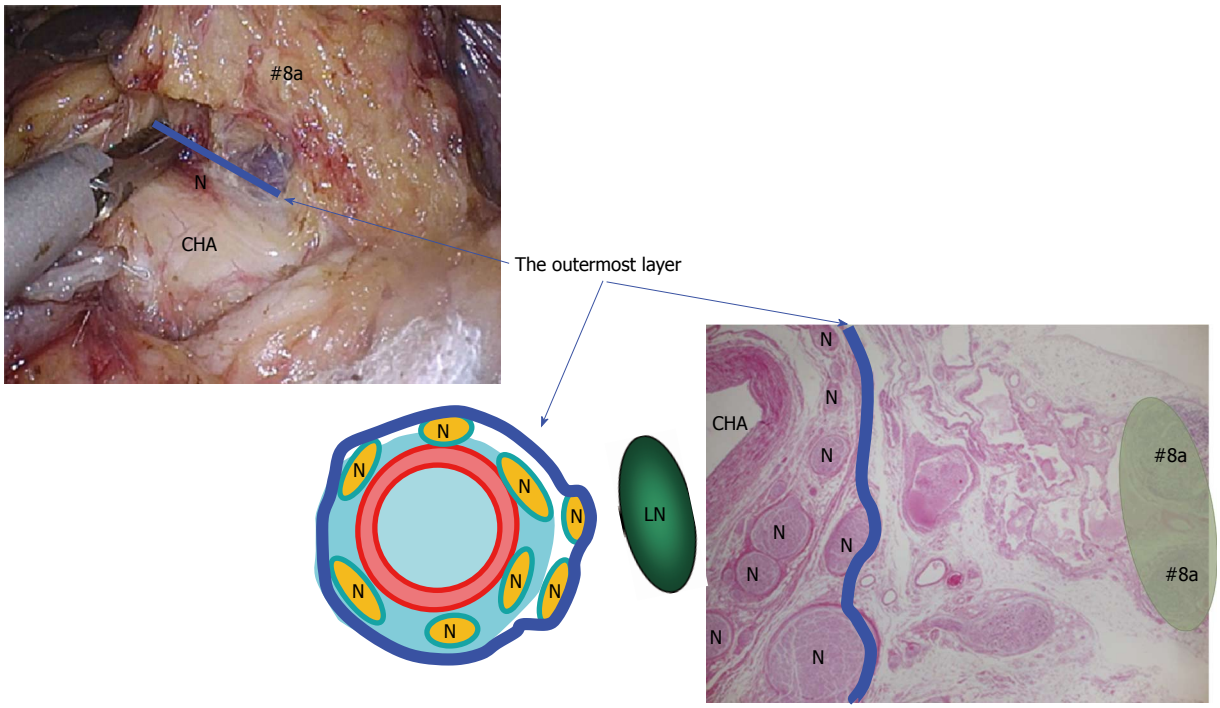

Figure 1 Outermost layer of the autonomic nerve. Shown in the blue line, lies between the vascular sheath of the major arteries and the fat tissue including lymph nodes. Appropriate tension given to this thin loose connective tissue layer generates sufficient space for safe, adequate and reproducible prophylactic lymph node dissection along the major arteries. LN: Lymph node; N: Nerve; CHA: Common hepatic artery.

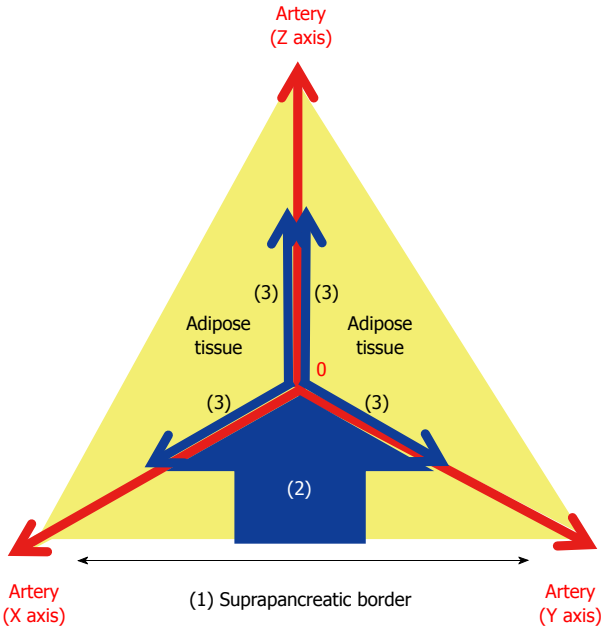

Figure 2 XYZ-axis theory. The following three steps result in effective probing of the outermost layer: (1) dissection of the serosal membrane on the suprapancreatic border; (2) dissection of the fat tissue in the caudo-cranial direction towards the zero point; and (3) dissection of the fat tissue bearing the target LNs in the medio-lateral direction along the outermost layer on the XZ and YZ axes. The outermost layer adjacent to the zero point should be exposed during the 2nd step.

more than 1500 LGs. At present, the standard type of operation for curable GC at our institute is totally laparoscopic D2 gastrectomy[5].

Suprapancreatic lymph node dissection: Outermost-layer oriented medial approach: D2 dissection entails removal of the LNs in the suprapancreatic area in distal and total gastrectomy[4]. Dissection of this area is technically demanding due to the serious risk of bleeding and/or pancreatic leakage derived from a major vessel or organ injury[49,50]. To improve the safety, efficacy, and reproducibility of suprapancreatic LN dissection, we developed our original methodology called outermost layer-oriented medial approach[46,50]. In this approach, the thin loose connective tissue layer between the autonomic nerve sheaths of the major arteries and the adipose tissue bearing lymphatic tissue is dissected[46,50]. We termed this layer as the outermost layer of the autonomic nerve (Figure 1)[46]. To identify this layer throughout the dissection process, we developed an original surgical theory, "XYZ-axis" theory (Figure 2), consisting of the following three steps: (1) cut the serosal membrane on the suprapancreatic border; (2) dissect suprapancreatic adipose tissue caudocranially towards the junction of the three arteries (zero point) to find the outermost layer; and (3) dissect the target adipose tissue mediolaterally along the outermost layer spreading on the XZ and YZ axes. Using this theory, the outermost layer could easily be found not only at the junction of left gastric, common hepatic, and splenic arteries (Figure 3A), but also at that of gastroduodenal, right gastroepiploic, and anterior superior pancreaticoduodenal arteries (Figure 3B) and that of proper hepatic and right gastric arteries (Figure 3C).

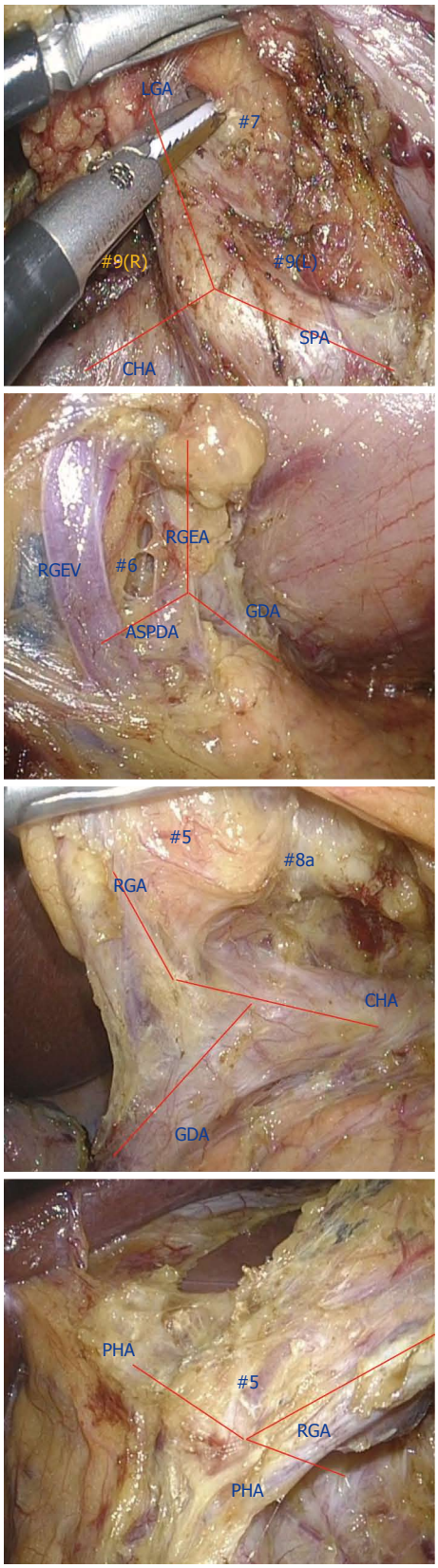

Figure 3 Lymph node dissection along the outermost layer using the XYZ-axis theory. A: No. 7 and 9 dissection, B: No. 6 dissection, C: No. 5 dissection.

LTG for AGC: Splenic hilar lymph node dissection: According to the JGCA guidelines, D2 total gastrectomy is recommended for advanced proximal GC[3,4]; however, as mentioned before, D2 lymphadenectomy combined with splenectomy or pancreaticosplenectomy has been reported to increase morbidity and mortality[5,51,52]. Therefore, the practical importance of station 10 LN dissection and splenectomy in D2 total gastrectomy is controversial[6-8].

We started totally LTG (TLTG) for AGC in 1997[47] and have established a stable and robust methodology, including splenic hilar LN (SHLN) dissection, even though LTG but not LDG has still been one of the independent risk factors for postoperative complications of LG[53,54]. Regarding the extent of SHLN dissection, D2 lymphadenectomy combined with distal pancreaticosplenectomy (D2 + PS) is performed in patients with tumors infiltrating into the pancreatic body or tail. D2 lymphadenectomy combined with splenectomy (D2 + S) is performed in patients with LN metastasis at the station 11 d or 10 or in those with greater curvature invasion. Spleen-preserving D2 lymphadenectomy (D2-S) is performed in patients with tumor depths cT $\geqslant$ 3 without LN metastasis at the station 11 d or 10, whereas D2 lymphadenectomy with preservation of station 10 LNs and the spleen (D2-10) is performed in patients without greater curvature invasion and with tumor depths cT2 (Figure 4)[55].

Regarding the operating procedures, additional care to control the extent of SHLN dissection in TLTG was given to: (1) the layer on the fusion fascia at the infrapancreatic border of the pancreatic tail; (2) the layer on the subretroperitoneal fascia on the left diaphragmatic crus around the upper pole of the spleen; and (3) the outermost layer of the splenic artery. Using these layers, the aforementioned four different types of SHLN dissection could easily be performed. Procedural details are summarized in our previous literature[55]. In this previous study, multivariate analysis revealed that operative time was the only significant factor associated with postoperative complications. Operative time, morbidity, and pancreatic fistula increased with increasing extent of SHLN dissection. Therefore, the extent of SHLN dissection should be appropriately attenuated if this is allowed by oncological factors. At present, according to the latest JGCA guidelines[4], complete clearance of station 10 nodes by splenectomy should still be considered for potentially curable T2-4 tumors invading the greater curvature of the upper stomach. However, in patients with T2-4/N0-2/M0 GC not invading the greater curvature, the JCOG0110 trial demonstrated that prophylactic splenectomy should be avoided to improve operative safety and survival[2,56].

Intracorporeal anastomosis: To fully utilize the advantages of LG, totally laparoscopic gastrectomy with intracorporeal anastomosis is promising. We have preferred intracorporeal anastomosis with linear staplers because of its handy, quick visible, and reproducible natures. In LDG, we have used delta-shaped anastomosis for Billroth-Ⅰ reconstruction[57-59],

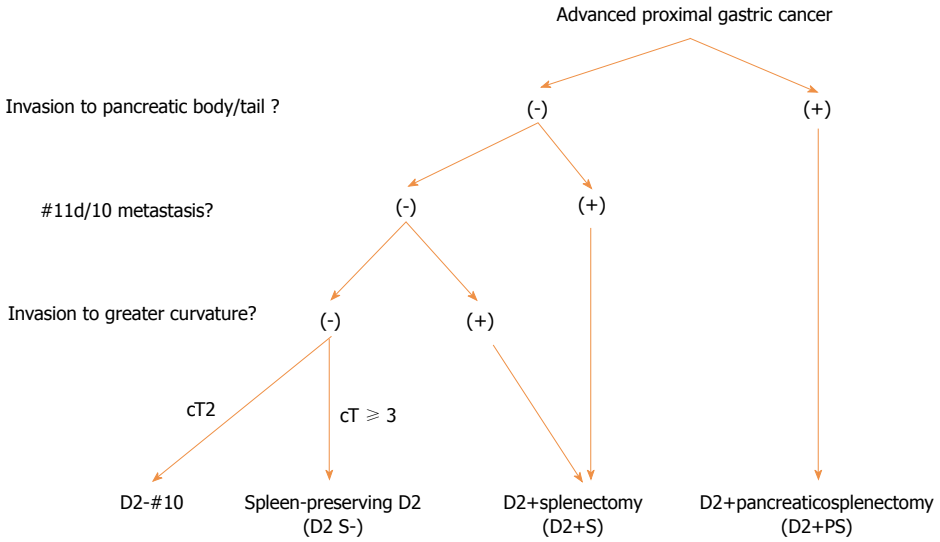

Figure 4 Indication for splenic hilar lymph node dissection at FHU.

antiperistaltic side-to-side anastomosis for Billroth-Ⅱ reconstruction, and functional end to end anastomosis for Roux-en-Y reconstruction[5]. In total gastrectomy, we have used functional end to end anastomosis[36] and overlap method[35] for intraabdominal and intrathoracic esophagojejunostomy, respectively. In proximal gastrectomy, modified overlap method with no-knife stapler has been used[60]. The details on intracorporeal anastomosis in LG are well summarized in the review article by Hosogi *et al*[60].

Outcomes: The short-term and long-term outcomes of LG for AGC at our institute have been satisfactory from both technical and oncological point of view (LG *vs* OG: mortality, 1.1% *vs* 0%, $P = 0.519$; morbidity, 24.2% *vs* 28.5%, $P = 0.402$; 5-year disease free survival, 65.8% *vs* 62.0%, $P = 0.737$; overall survival, 68.1% *vs* 63.7%, $P = 0.968$). Details are demonstrated in our previous reports[38,55].

Robotic gastrectomy

In Japan, da Vinci S HD Surgical System received approval by the Drugs, Cosmetics and Medical Instruments Act in November, 2009. We introduced da Vinci S to our institution in 2009 for the first time in our county, and have been actively using this system for operable patients with resectable upper GI cancer who agreed to uninsured use of the robot[46,53,61].

According to the latest meta-analysis of robotic gastrectomy (RG) *vs* LG, combining the findings from previous observational studies with small sample size, use of the robot significantly increased operative time and cost, whereas there were no significant differences in other short-term outcomes including blood loss, number of dissected lymph nodes, surgical margins, postoperative complications and duration of hospital stay[62]. The only large non-randomized prospective study (NCT01309256), recently reported from Korea, demonstrated similar outcomes[63]. These reports suggested that use of the robot might even deteriorate the cost-effectiveness[62,63]. In other words, the greatest issue around RG is a lack of clear benefits of the robotic system which corroborate the longer duration of operation and higher cost[63]. However, most of the patients enrolled in these previous studies had pathological stage Ⅰ diseases, and these studies failed to eliminate the learning effect or the selection bias at least partly generated by more expensive copayment in RG[62,63]. The impact of RG on long-term outcomes has largely been unclear[64,65]. Thus, the advantages of RG for AGC conducted by fully-trained robotic surgeons have never been clarified. In addition, several reports have demonstrated the short learning curve of RG[66-69].

Since 2009, we have performed RG for more than 250 patients not only with EGC but with AGC. Then, according to our retrospective analyses in comparison with LG (EGC *vs* AGC, 57% *vs* 43%), RG reduced morbidity down to one fifth including pancreatic fistula, leading to further improvement in short-term postoperative courses, although it slightly increased blood loss and operative time[53,70]. Multivariate analyses clearly demonstrated that conventional LG (non-use of the surgical robot), total gastrectomy (*vs* distal) and D2 lymphadenectomy (*vs* D1+) were the significant independent risk factors determining postoperative complications[53]. Moreover, the greater the extent of gastric resection and LN dissection, the more effective the use of the robot[53]. In terms of long-term results, 3-year overall survival did not change between RG and LG[71]. Not only oncological factors including tumor size and clinical stage but also surgical factors including pancreatic fistula were found to be associated with three-year recurrence free survival, indicating the oncological as well as surgical importance of preventing pancreatic fistula[71,72]. These

data suggest that the best indication for the use of the robot might be RG for AGC with D2 dissection[53]. Thus, multi-institutional prospective studies conducted by experienced robotic surgeons, in which considerable number of patients with AGC are enrolled, should be required to determine whether use of the robotic system for AGC truly attenuates pancreatic fistula, possibly leading to improvement in long-term outcomes[70].

Since the beginning of October, 2014, we have been conducting a multi-institutional single-arm prospective study (UMIN000015388), which Japanese Ministery of Health, Labor, and Welfare has recently approved for Advanced Medical Technology ("senshiniryo")[70]. This study was designed to determine the impact of the use of the robot, for minimally invasive radical gastrectomy to treat resectable GC, on short-term outcomes, mainly focusing on postoperative complications, as well as long-term outcomes and cost. The specific hypothesis of this study was that the use of the robot in patients with cStage Ⅰ or Ⅱ diseases reduces the morbidity (Clavien-Dindo Classification Grade ≥ Ⅲ[73]) of 6.4% in conventional LG down to 3.2%. The sufficient sample size was calculated to be 330. All the patients will be registered in 2 years after starting this study and followed up for 3 years, thus the expected study period should be 5 years in total. Interim analyses will be done once the initial 220 cases are registered. As of January 31, 2016, 122 patients from 5 institutions have been registered.

ESOPHAGEAL CANCER

History

Since 1992, when Cuschieri *et al*[74] first reported on VATS-E, many groups have described various methods[75-79]. In Japan, Akaishi *et al*[75] first reported on thoracoscopic total esophagectomy with en bloc mediastinal lymphadenectomy in 1996. Kawahara *et al*[76] demonstrated the details of VATS-E with extended lymphadenectomy in 1999, and Osugi *et al*[80] clarified the long-term outcomes of VATS-E. We performed prone VATS-E with CO2 insufflation in 2006 for the first time in our country[61].

Indication

The indication for VATS-E is relatively wider than that for LG[10]. VATS-E has currently been applied up to locally advanced EC even after neoadjuvant chemoradiotherapy[9,10]. Only some conditions including T4 tumor, severe intrathoracic adhesion, and one-lung ventilation failure are considered to be excluded from the indication for VATS-E[10,77,81].

Left lateral decubitus vs prone position

Regarding the patient positions used for VATS-E, similar to right transthoracic open esophagectomy (OE), the left lateral decubitus position had been mostly used[75,82]. However, the prone position has increasingly been used recently[74,83-85]. Prone VATS-E is characterized by operating surgeon-friendly sense of use brought by more ergonomic set up as well as a drier operative field given by gravity in combination with the positive intrathoracic pressure generated by CO_2 insufflation[61]. To enjoy these advantages of the prone position as well as those of laparoscopic horizontal magnified view with overcoming the laparoscopic limited range of motion, we fully mobilize the "meso-oesophagus"[86] from lower up to upper mediastinum prior to the LN dissection of station 106 recR, 106 recL+tbL, and 112 (Japanese Classification of Esophageal Cancer, the 11th ed[87]) using the 6-trocar system in the hemi-prone position (Figure 5).

Outcomes

To date, a number of single-institution studies have demonstrated acceptable short-term outcomes of VATS-E for thoracic EC regarding operative time, blood loss and postoperative complications, which are comparable with those of conventional OE[13,81]. According to a meta-analysis based on these small case-control studies, VATS-E reduced blood loss, total morbidity and respiratory complications, leading to shorter intensive care unit and hospital stay in comparison with OE[88-90]. In terms of long-term outcomes, a limited number of case-control studies have demonstrated the comparable results with conventional OE[80,89,91,92]. Therefore, to determine the feasibility and beneficial effects of VATS-E, multicenter prospective RCTs are warranted.

ECOG2202: The Eastern Cooperative Oncology Group (ECOG) performed the first prospective phase Ⅱ multicenter trial (ECOG2202[93]) to assess the feasibility of VATS-E. A total of 110 patients were enrolled at 17 credentialed sites. The primary endpoint was 30-d mortality. 30-d and perioperative mortality was 2.1% and 2.9%, respectively. Grade 3 or 4 (CTCAE v3.0[27]) adverse effects occurred in 49.5% of the eligible 105 patients. Estimated 3-year overall survival was 58.4% (95%CI: 47.7%-67.6%). These data suggested that VATS-E was feasible and safe with acceptable perioperative and oncological outcomes.

Traditional Invasive *vs* Minimally Invasive Esophagectomy trial: Traditional Invasive *vs* Minimally Invasive Esophagectomy (TIME) trial is the first multicenter RCT comparing short-term outcomes of prone VATS-E and those of OE, which was conducted by a study group in Europe[77,94]. In this study, 56 and 59 patients were randomly assigned to the OE and the VATS-E group, respectively. As a result, VATS-E reduced, intraoperative blood loss, postoperative pain, postoperative pulmonary infection, and vocal cord palsy, leading to reduced hospital stay and improved postoperative QoL. No siginificant difference was observed in mortality and the number of

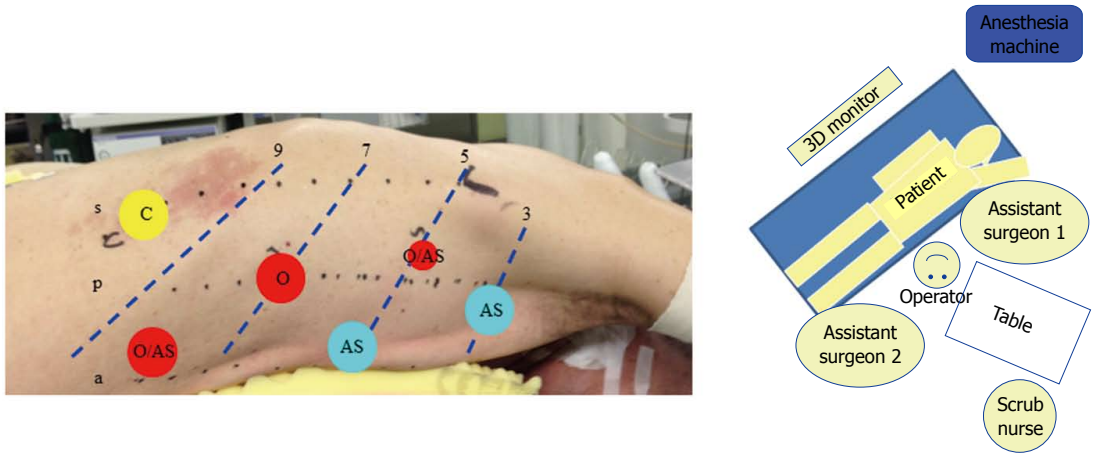

Figure 5 Setup for prone VATS-E at Fujita Health University. A: The patient in the hemi-prone position using the six-trocar system. 12 mm trocars are used except for the trocar in the 5th intercostal space (ICS) behind the posterior axillary line; B: OR setup. s: Scapula angle line; p: Posterior axillary line; a: Anterior axillary line; 3: 3rd ICS; 5: 5th ICS; 7: 7th ICS; 9: 9th ICS; O: Operating surgeon; AS: Assistant surgeon.

dissected lymph nodes. These findings suggested the advantages of VATS-E over OE in terms of short-term outcomes.

Robotic esophagectomy

The robotic esophagectomy has been less commonly performed than robotic gastrectomy. Thus, the impact of the use of D*VSS* on esophagectomy has been assessed mostly in case-series with small sample size[61,95-109]. Various groups have reported on feasibility and safety with good short-term outcomes in a wide-range of approaches to esophagectomy[110]. Van der Sluis *et al*[111] have reported sufficient oncological long-term outcomes of robotic esophagectomy for advanced esophageal cancer (5-year overall survival, 42%). Hernandez *et al*[100] demonstrated that the learning curve for a robotic-assisted procedure appears to begin near proficiency after 20 cases for surgeons experienced in conventional minimally invasive approach. Further studies are warranted to determine advantages and disadvantages of robotic esophagectomy.

Since 2009, we have performed robotic radical esophagectomy in the prone position for more than 40 patients. Then, compared to conventional MIS, robotic approach significantly reduced incidence of vocal cord palsy and hoarseness, suggesting that the use of the robot, which promotes more accurate recurrent laryngeal nerve identification and dissection[95], should reduce the chances of recurrent laryngeal nerve injury, resulting in preserved laryngopharyngeal function[61].

DISCUSSION

Although MIS for upper GI cancer has consistently appeared to improve short-term outcomes and at least preserve long-term outcomes, solid evidences that verify feasibility of MIS and even superiority to open surgery have still been lacking. Advantages and disadvantages of LDG for EGC and AGC over ODG will soon be clarified after the ongoing multicenter RCTs are concluded, however, those of LTG and VATS-E will have been unclear for the time being.

One of the principal reasons why surgeons have been attracted to MIS must be the laparoscopically enhanced anatomy provided by the magnified vivid image with high definition in combination with the horizontal view. To fully utilize these advantages of MIS, the disadvantages of MIS including limited range of motion has to be overcome. We believe one of the solutions may be the laparoscopic manipulation in the caudocranial and/or mediolateral manner, and another may be the use of the surgical robot as indicated in our previous reports[46,50,53,55,61].

We wish to further develop MIS for advanced cancer and that requiring advanced skills by actively utilizing novel technologies including the surgical robot, based on the principles and methods grown through conventional MIS and open surgeries.

In conclusion, technical advancements and development of endoscopic instruments will continue to evolve MIS for upper GI cancer. The outcomes should be validated in a scientific fashion.

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TOPIC HIGHLIGHT

2016 Gastric Cancer: Global view

Recent updates of precision therapy for gastric cancer: Towards optimal tailored management

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Abstract

Signaling pathways of gastric carcinogenesis and gastric cancer progression are being avidly studied to seek optimal treatment of gastric cancer. Among them, hepatocyte growth factor (HGF)/c-MET, phosphoinositide 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) and janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) pathways have been widely investigated. Their aberrant expression or mutation has been significantly associated with advanced stage or poor prognosis of gastric cancer. Recently, aberrations of immune checkpoints including programmed cell death-1/programmed cell death ligand-1 (PD-1/PD-L1) have been suggested as an important step in the formation of a microenvironment favorable for gastric cancer. Accomplishments in basic research have led to the development of novel agents targeting these signaling pathways. However, phase Ⅲ studies of selective anti-HGF/c-MET antibodies and mTOR inhibitor failed to show significant benefits in terms of overall survival and progression-free survival. Few agents directly targeting STAT3 have been developed. However, this target is still critical issue in terms of chemoresistance, and SH2-containing protein tyrosine phosphatase 1 might be a significant link to effectively inhibit STAT3 activity. Inhibition of PD-1/PD-L1 showed durable efficacy in phase Ⅰ studies, and phase Ⅲ evaluation is warranted. Therapeutic strategy to concurrently inhibit multiple tyrosine kinases is a reasonable option, however, lapatinib needs to be further evaluated to identify good responders. Regorafenib has shown promising effectiveness in prolonging progression-free survival in a phase Ⅱ study. In this topic highlight, we review the biologic roles and outcomes of clinical studies targeting these signaling pathways.

Key words: Gastric cancer; Hepatocyte growth factor; Mammalian target of rapamycin; Signal transducer and activator of transcription 3; Programmed cell death ligand-1

Core tip: Among various cellular signaling pathways, hepatocyte growth factor/c-MET, phosphoinositide 3-kinase/Akt/mammalian target of rapamycin and janus kinase 2/signal transducer and activator of transcription 3 pathways are reportedly important in gastric carcinogenesis and metastasis. Aberrations of immune checkpoints have been vigorously investigated. However, clinical results of their target agents have not always matched the theoretical expectations of efficacy. In this review, we summarize the biologic impacts of the aforementioned signaling pathways, and their recent clinical outcomes including those of multiple kinase inhibitors in gastric cancer.

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INTRODUCTION

Gastric cancer is the fourth common malignant tumor worldwide, and the second most common cause of cancer-related mortality[1]. The progress in therapeutic approaches has allowed complete remission of early gastric cancer by surgical or even endoscopic resection of tumors. However, if gastric cancer is advanced when diagnosed, the prognosis is generally poor and survival time is short even after surgical complete resection. Therefore, highly selective and effective chemotherapy remains an important issue for appropriate management of advanced gastric cancer.

A recent notable study provided a comprehensive molecular evaluation of primary gastric adenocarcinoma tissues as part of The Cancer Genome Atlas project[2]. The authors proposed four subtypes of gastric cancer according to the molecular characteristics: Epstein-Barr virus positive tumors, microsatellite instability tumors, genomically stable tumors and chromosomal unstable tumors. This study is a prime example of efforts to develop optimal classification of gastric cancer by analyzing common dysregulated pathways and to provide distinct tailored therapy for individual patients. Since the report of significant clinical benefits of trastuzumab in human epidermal growth factor receptor 2 (HER2)-positive gastric and esophagogastric junction (EGJ) adenocarcinoma[3], various targets have been investigated in the treatment of advanced gastric cancer. These targets include epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), hepatocyte growth factor(HGF)/c-MET and mammalian target of rapamycin (mTOR)[4]. However, we still have a long way to go before complete conquest of gastric cancer.

In this topic highlights, we aimed to review the biologic roles of several molecular signaling pathways on the basis of recent trials of targeted therapies in advanced gastric cancer. These include the HGF/c-MET, phosphoinositide 3-kinase (PI3K)/Akt/mTOR, janus kinase 2/signal transducer and activator of transcription 3 (JAK2/STAT3) and programmed cell death-1/programmed cell death ligand-1 (PD-1/PD-L1) pathways. In the latter part, we focus on clinical outcomes of newly developed agents targeting the aforementioned pathways and summarize the findings of some clinical studies of multi-kinase inhibitors (MKIs), which can simultaneously multiple receptor tyrosine kinases (RTKs) in advanced gastric cancer.

CELLULAR SIGNALING PATHWAYS OF GASTRIC CANCER

HGF/c-MET pathway

c-MET is a heterodimeric subfamily of RTK. c-MET is composed of an α-chain, which possesses only an extracellular domain, and a β-chain composed of extracellular, transmembrane and intracellular domains[5]. The ligand of c-MET, HGF, is converted into an active form that causes dimerization and activation of the c-MET receptor. The activated HGF/c-MET signal leads to autophosphorylation of multiple tyrosine residues of the intracellular region of c-MET, such as Y1230, Y1234, Y1235, Y1349 and Y1356, which form multi-functional docking sites to recruit several intracellular adaptor proteins[6]. Among them, Grb2-associated binder 1 (GAB1) can directly bind to c-MET or forms a complex with growth factor-bound protein 2 (GRB2) to indirectly interact with c-MET. The c-MET association recruits several main adaptor proteins including STAT3 and PI3K, which in turn activate downstream biologic effects including cellular proliferation, migration/invasion and induction of epithelial-mesenchymal transition (EMT) (Figure 1)[7].

Clinical impact of the HGF/c-MET pathway in gastric cancer has been well documented. High protein expression rate of c-MET in gastric carcinoma tissue has been demonstrated by immunohistochemistry (IHC; 43%-82%)[8,9] and by gene amplification rate (2%-10%)[9-12]. In both approaches, the elevated expression of c-MET has been positively associated with advanced tumor stage and poor survival rate. Consequently, multiple agents targeting the HGF/c-MET signaling pathway are being evaluated. Tivantinib, an anti-c-MET tyrosine kinase inhibitor (TKI), used in combination with the EGFR TKI, erlotinib, has extended progression-free survival (PFS) in patients

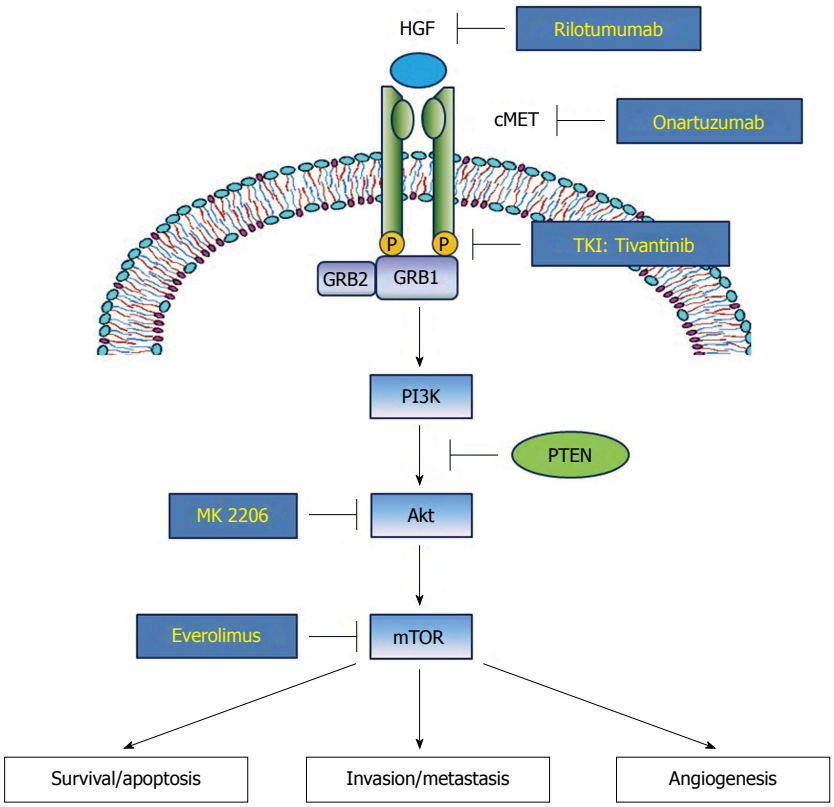

Figure 1 Hepatocyte growth factor/mesenchymal epithelial transition factor and phosphoinositide 3-kinase/Akt/mammalian target of rapamycin pathway, and their inhibitors evaluated in gastric cancer patients. HGF: Hepatocyte growth factor; cMET: Mesenchymal epithelial transition factor; GAB1: Grb2-associated binder 1; GRB2: Growth factor-bound protein 2; PI3K: Phosphoinositide 3-kinase; PTEN: Phosphatase and tensin homolog; mTOR: Mammalian target of rapamycin.

with locally advanced or metastatic non-squamous non-small-cell lung cancer in a phase Ⅲ trial[13]. In gastric cancer, phase Ⅲ studies for rilotumumab, an anti-HGF monoclonal antibody, and onartuzumab, an anti-c-MET monoclonal antibody, have been completed, and clinical outcomes of tivantinib were recently reported[14].

PI3K/Akt/mTOR pathway

The PI3K-Akt-mTOR pathway plays a pivotal role in oncogenesis and progression including cell growth, survival, invasion/metastasis and angiogenesis, and gastric cancer is no exception. PI3K is usually activated through binding and stimulation of various RTKs by growth factors including HGF and c-MET. Activated PI3K subsequently phosophorylates and activates phosphatidylinositol 3,4-biphosphate (PIP2), phosphatidylinositol 3,4,5-triphosphate (PIP3), phosphoinositide-dependent protein kinase 1 and Akt[15]. Akt, which is also termed protein kinase B, is a major effector protein of the PI3K pathway. Phosphorylated Akt (p-Akt) modulates various biologic functions like cell survival, migration/invasion and angiogenesis through downstream adaptor molecules including mTOR (Figure 1)[16].

Genetic alteration of biological signals involving the PI3K/Akt/mTOR pathway has been frequently detected in gastric carcinoma. For example, a point mutation of *PIK3CA* encoding p110 (a class IA subunit of PI3K) is often observed in gastric carcinoma tissues, ranging from 4.3%-25%[17-21], with the point mutation mostly seen in exon 9 and exon 20[17]. Their mutation or gene amplification is positively associated with the T stage of gastric cancer[20,22]. In contrast, *PTEN*, which encodes phosphatase and tensin homolog and inactivates Akt by converting PIP3 to PIP2, is deleted in 4%-23% of gastric cancers[21,23,24] and loss of heterozygosity (LOH) is observed in 17%-47% cases of gastric cancer. LOH of PTEN is significantly associated with p-Akt level in gastric carcinoma tissues, TNM stage and poor prognosis of survival[25-29]. Activated Akt signaling promotes mTOR protein complexes 1 and 2 (mTORC1 and mTORC2), which can play pivotal roles in cancer cell migration and metastasis. Prevalence of mTOR expression is reported as approximately 50% in gastric cancer tissues, and is negatively associated with PTEN expression[30,31].

Clinical and laboratory evidence indicates the promising potential of targeting the PI3K-Akt-mTOR signaling pathway for efficacious treatment of gastric cancer, and various kinds of inhibitors or antibodies acting on this pathway have been developed and tried. These inhibitors are classified into several categories that include PI3K inhibitors, dual mTOR1/mTOR2 inhibitors, Akt inhibitors, mTOR1 inhibitors and dual PI3K/mTOR inhibitors[15]. Among them, a phase Ⅰ study

of isoform specific PI3K inhibitor (p110α) BYL719 is ongoing (NCT01613950)[32], and clinical outcomes of Akt inhibitor MK 2206 and mTOR1 inhibitor everolimus and rapamycin were previously reported, and are dealt with more fully in the latter part of this review.

JAK2/STAT3 pathway and inhibitory role of SH2-containing protein tyrosine phosphatase 1

The most established stimulator of STAT3 signaling pathway is the interleukin (IL)-6 family that includes IL-6, IL-11 and leukemia inhibitory factor, which bind to their receptors, and then phosphorylate and activate JAK2. Activated JAK2 recruits and activates STAT3 by phosphorylation, which can dimerize and translocate into the nucleus to act as a transcription factor and up-regulate various target genes involving cellular proliferation, migration/invasion and angiogenesis[33]. Indeed, persistent constitutive activation of JAK2/STAT3 in cancer cells is closely associated with gastric carcinogenesis and poor prognosis[34]. Besides this classic effect of JAK2/STAT3 pathway in cancer development, another pivotal role of STAT3 protein is the tumor microenvironment, where immune cells can be recruited and STAT3 can mediate various interactions with cancer cells to generate tumor progression. In gastric cancer, *Helicobacter pylori* (*H. pylori*)-induced cytotoxin-associated antigen (CagA) is closely associated with STAT3 activity in both gastric epithelial cells and mucosal immune cells. For example, *H. pylori* infection and CagA secretion can lead to IL-23 release from dendritic cells, which binds to their receptor and activates JAK2/STAT3 transmembrane signaling of naïve $CD4^+$ T-cells, and causes differentiation of T-helper (Th)-17 specific lineages to release associated cytokines including IL-17[35]. Up-regulated IL-17 can promote pro-inflammatory and oncogenic environment. Expression level of IL-17 is positively correlated with depth of tumor, lymphovascular invasion and lymph node involvement in gastric cancer tissues[36,37], and IL-17 mediates angiogenesis *via* up-regulation of VEGF *in vivo* and *in vitro*[38]. In gastric epithelial cells, CagA is translocated *via* the type-Ⅳ secretion system and releases IL-11. The released IL-11 bind to their receptor and activate the JAK2/STAT3 cascade[39]. Activated STAT3 functions as a transcription factor to induce many target genes involved in proliferation, invasion/metastasis and angiogenesis including cyclin D1, surviving, matrix metalloproteinase-9, CD44v6 and VEGF[34,40].

Thus, a therapeutic strategy to target the STAT3 signaling pathway appears to be reasonable. Routes of inhibition include blockade of JAK activation by dephosphorylation, inhibition of STAT3 phosphorylation, dimerization or gene transcription[35]. In terms of dephosphorylation, several phosphatases have been reported to be associated with STAT3 activity. Among them, SH2-containing protein tyrosine phosphatase 1 (SHP1) may be crucial in the down-regulation of the JAK2/STAT3 pathway by dephosphorylation[41-43]. Several candidate agents including natural compounds were reported to induce SHP1 and inhibit STAT3 activity. Sorafenib and its synthetic analogues also can act as a SHP1 agonist to inhibit phosphor-STAT3 activity and show various anti-cancer effects, such as promotion of apoptosis, overcoming of radio- or chemo-resistance and inhibition of EMT or fibrosis on hepatocellular carcinoma cell lines[44-51]. However, the exact inhibitory role of SHP1 in gastric cancer development and progress is unknown. We recently showed that expression of SHP1 is reduced or ameliorated in various gastric cancer cell lines due to epigenetic silencing, and that reinforced SHP1 expression significantly inhibits cellular proliferation, migration/invasion and induce apoptosis[52]. SHP1 might be a promising target to effectively inhibit JAK2/STAT3 activity in gastric cancer cells (Figure 2).

Immune checkpoints

Immune checkpoints regarding tumor infiltrating lymphocytes and immune evasion mechanism associated with carcinogenesis have been studied in the search for alternative therapeutic targets. Among them, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and PD-1, which are minimally expressed on the surface of resting T-lymphocytes but are widely expressed on activated T-lymphocytes, have been intensively studied for gastric carcinogenesis, and anti-PD-1 antibodies are already in clinical trials of gastric cancer chemotherapy[53]. Ligands for PD-1 (PD-L1) and CTLA-4 (B7-1/B7-2), which are expressed on the surface of tumor cells, bind to PD-1 and CTLA-4 respectively, inhibit pivotal function of effector T-cells for immune surveillance and consequently promote the growth of gastric cancer cells (Figure 3)[54].

PD-1 expression differs between gastric cancer tissues and non-cancerous tissues, with the significantly up-regulated PD-1 level in gastric cancer tissues being significantly correlated with poor clinical parameters including increased tumor size, advanced stage, metastasis and patient survival[55-58]. Furthermore, PD-1 expression on $CD4^+$ and $CD8^+$ T cells from gastric cancer tissues is higher than non-cancer tissues or peripheral blood mononuclear cells from normal subjects, and is significantly associated with disease progression[59]. However, a recent Korean study demonstrated that expression rate of PD-L1 on gastric cancer tissues was 43.6%, and was related to less advanced stage, intestinal type, well/moderately differentiated adenocarcinoma rather than poor differentiation and better overall survival (OS) and disease-free survival[60]. A recent Chinese study investigated PD-L1 expression level using large number of gastric cancer tissues (almost 400 specimens); PD-L1 expression was significantly associated with TIL density, and moderate to high TIL density was closely correlated with better prognosis[61]. Thus, the

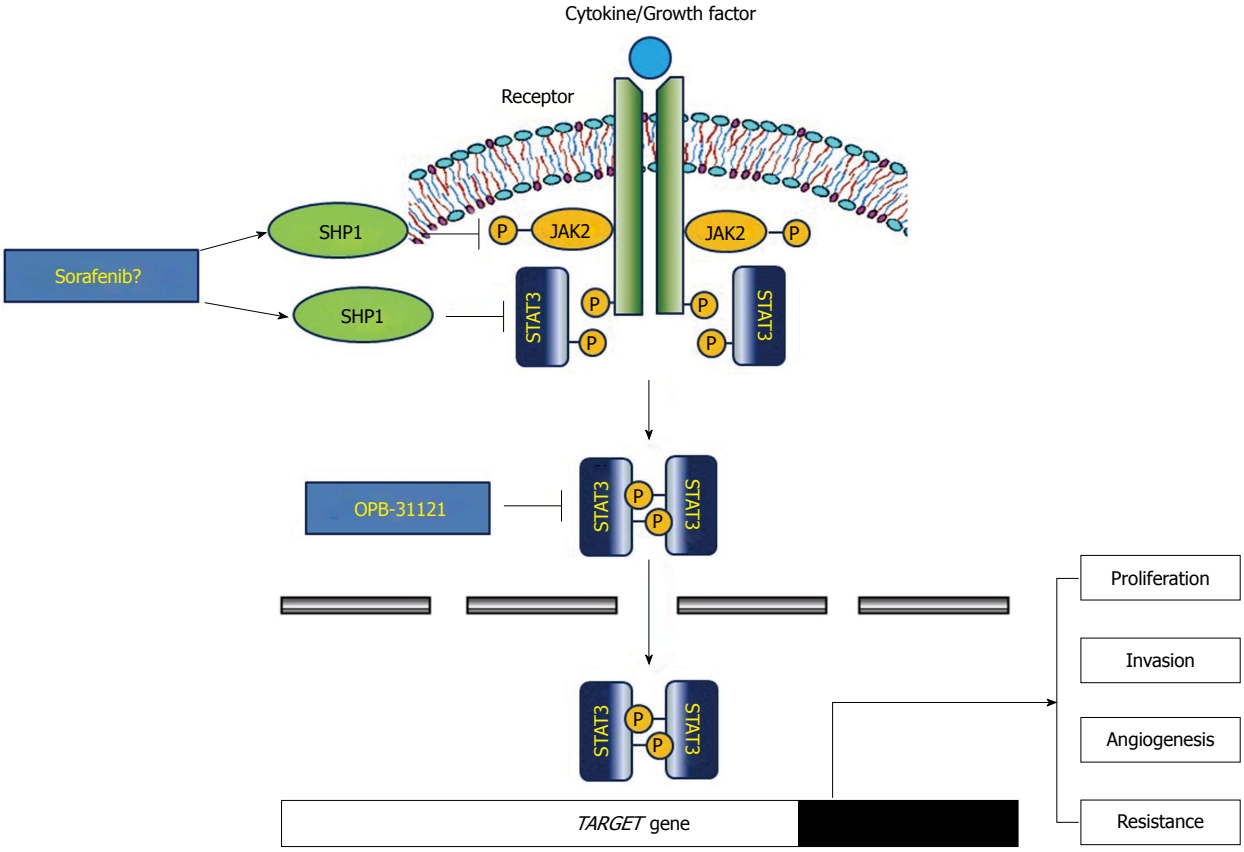

Figure 2 Janus kinase 2/signal transducer and activator of transcription 3 pathway and inhibitory role of SH2-containing protein tyrosine phosphatase 1. JAK2: Janus kinase 2; STAT3: Signal transducer and activator of transcription 3; SHP1: SH2-containing protein tyrosine phosphatase 1.

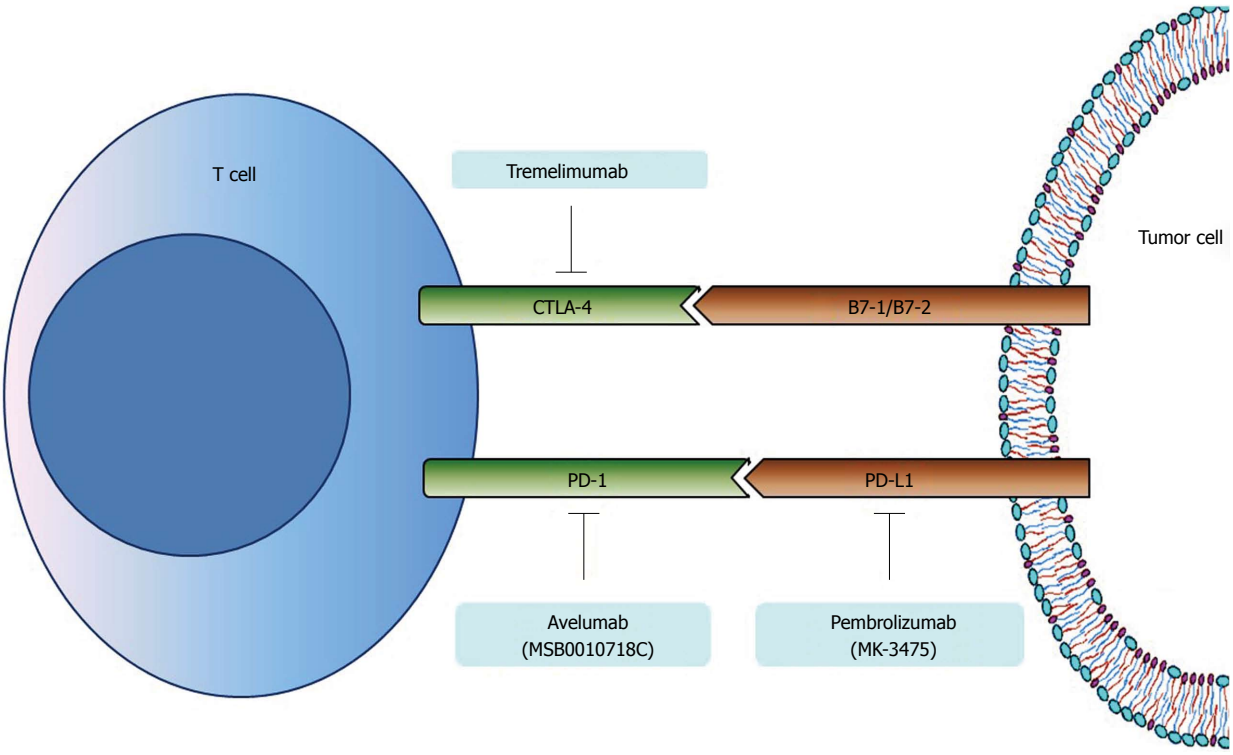

Figure 3 Immune checkpoints on tumor cell and T-cell, and their monoclonal antibodies evaluated in gastric cancer patients. CTLA-4: Cytotoxic T-lymphocyte-associated protein 4; PD-1: Programmed cell death-1; PD-L1: Programmed cell death ligand-1.

Table 1 Clinical outcomes of recent trials of targeted therapy in advanced gastric and esophagogastric junction adenocarcinoma

Author and trial	Line of treatment	Phase of study	*n*	Treatment arms	Outcomes
Anti-HGF/cMET antibodies					
Cunningham *et al*[63], RILOMET-1 (2015)	First	Ⅲ	609	ECX + rilotumumab *vs* ECX + placebo	OS: 9.6 mo *vs* 11.5 mo (HR = 1.37, *P* = 0.016) PFS: 5.7 mo *vs* 5.7 mo (HR = 1.30, *P* = 0.016) ORR: 30% *vs* 39.2% (OR 0.67, *P* = 0.027)
Shah *et al*[65], METGastric (2015)	First	Ⅲ	562	mFOLFOX + ornatuzumab *vs* mFOLFOX + placebo	OS: 11.0 mo *vs* 11.3 mo (HR = 0.82, *P* = 0.244) PFS: 6.7 mo *vs* 6.8 mo (HR = 0.90, *P* = 0.429) ORR: 46% *vs* 41% (*P* = 0.253)
Malka *et al*[66], PRODIGE 17 ACCORD 20 MEGA (2015)	First	Ⅱ	162	mFOLFOX alone *vs* mFOLFOX + panitumumab *vs* mFOLFOX + rilobumumab	4-mo PFS rate: 71 *vs* 63 *vs* 63% PFS: 5.8 mo *vs* 5.2 mo *vs* 7.6 mo ORR: 54% *vs* 44% *vs* 50%
Akt/mTOR inhibitors					
Hudis *et al*[68] (2013)	Second/third	Ⅰ	34	Trastuzumab + Akt inhibitor (MK-2206)	RR (including stable disease): 24% Time to progression: 72 d
Ohtsu *et al*[70] GRANITE (2013)	Second/third	Ⅲ	646	Everolimus *vs* BSC	OS: 5.4 mo *vs* 4.3 mo (HR = 0.90, *P* = 0.124) PFS: 1.7 mo *vs* 1.4 mo (HR = 0.66, *P* < 0.001) ORR: 4.5% *vs* 2.1%; DCR: 43.3% *vs* 22.0%
Shen *et al*[71] (2014)	First	Ⅱ	40	Everolimus + cisplatin + HDFL	OS: 10.5 mo (95%CI: 8.6-12.3) PFS: 6.9 mo (95%CI: 4.9-8.4)
STAT3 inhibitor					
Oh *et al*[74] (2015)	Second/third	Ⅰ	25	STAT3 inhibitor (OPB-31121)	RR (including stable disease): 44.4%
Immune checkpoints inhibitors					
Ralph *et al*[89] (2010)	Second	Ⅱ	18	Tremelimumab	OS: 4.8 mo (95%CI: 4.06-5.59) 12 mo OS rate: 33% (95%CI: 14-54) RR (including stable disease): 27.8%
Bang *et al*[90], KEYNOTE-012 (2015)	Second/third	Ⅰ	39	Pembrolizumab (MK-3475)	OS: 11.4 mo; PFS 1.9 mo ORR: 22% (95%CI: 10-39)
Yamada *et al*[93] (2015)	Second/third	Ⅰ	20	Avelumab (MS0010718C)	PFS: 11.9 wk (95%CI: 6.0-12.3) ORR: 15.0% (95%CI: 3.2-37.9)
Multikinase inhibitors					
Sun *et al*[95] (2010)	First	Ⅱ	44	Sorafenib + docetaxel + cisplatin	OS: 13.6 mo (90%CI: 8.6-16.1) PFS: 5.8 mo (90%CI: 5.4-7.4) PR: 41% (90%CI: 28-54)
Martin-Richard *et al*[96], GERCAD (2013)	First	Ⅱ	40	Sorafenib + oxaliplatin	OS: 6.5 mo (95%CI: 5.2-9.6) PFS: 3 mo (95%CI: 2.3-4.1) RR (including stable disease): 50.0%
Hecht *et al*[100], LOGiC (2015)	First	Ⅲ	487	CapeOx + lapatinib *vs* CapeOx + placebo	OS: 12.2 mo *vs* 10.5 mo (HR = 0.91, *P* = 0.349) PFS: 6.0 mo *vs* 5.4 mo (HR = 0.82, *P* = 0.0381) ORR: 53% *vs* 39% (*P* = 0.0031)
Satoh *et al*[101], TyTAN (2014)	Second	Ⅲ	261	Lapatinib + paclitaxel *vs* Paclitaxel alone	OS: 11.0 mo *vs* 8.9 mo (HR = 0.84, *P* = 0.1044) PFS: 5.4 mo *vs* 4.4 mo (HR = 0.85, *P* = 0.2441) ORR: 27% *vs* 9% (*P* < 0.001)
Pavlakis *et al*[103], INTEGRATE (2015)	Second/third	Ⅱ	147	Regorafenib *vs* placebo	OS: 5.8 mo *vs* 4.5 mo (HR = 0.74, *P* = 0.11) PFS: 2.6 mo *vs* 0.9 mo (HR = 0.40, *P* < 0.0001) RR (including stable disease): 44% *vs* 16%
Lee *et al*[106] (2015)	First	Ⅱ	66	CapeOx + pazopanib	PFS: 6.5 mo; OS: 10.5 mo; ORR: 57.6%

ECX: Epirubicin/Cisplatin/Capecitabine; mFOLFOX: 5-fluorouracil/leukovorin/oxaliplatin; OS: Overall survival; HR: Hazard ratio; PFS: Progression free survival; ORR: Objective response rate; BSC: Best supportive care; DCR: Disease control rate; HDFL: High-dose 5-fluorouracil/leucovorin; CapeOx: Capecitabine/oxaliplatin.

exact relationship between PD-1/PD-L1 expression and clinical parameters needs to be further evaluated.

RECENT TRIALS OF TARGET THERAPY FOR GASTRIC CANCER

The pivotal ToGA study of targeted therapy for the treatment of unresectable gastric/EGJ cancer investigated the synergistic effects of trastuzumab, a monoclonal anti-HER2 antibody, with capecitabine/cisplatin or fluorouracil/cisplatin regimen. OS and PFS were significantly prolonged[3]. Since then, various target agents have been tried for the optimal treatment of advanced gastric cancer. Among them, newly developed drugs targeting the HGF/c-MET, PI3K/Akt/mTOR, JAK2/STAT3 and PD-1/PD-L1 pathways are dealt with here, and MKIs that simultaneously target multiple tyrosine kinases is also introduced in this section. A portion of these studies were presented at the 2015 annual meeting of the American Society of Clinical Oncology (ASCO); their outcomes are summarized in Table 1.

Phase Ⅲ studies of anti-HGF/c-MET antibodies

Rilotumumab, an anti-HGF monoclonal antibody, and onartuzumab, an anti-c-MET monoclonal antibody,

have been tried as first-line treatments for gastric or EGJ adenocarcinoma in phase Ⅲ studies. Their clinical outcomes were presented at the ASCO 2015 meeting. Rilotumumab significantly increased PFS when combined with ECX (epirubicin/cisplatin/capecitabine) regimen in a phase Ⅰb/Ⅱ study[62]. From this background, the phase Ⅲ RILOMET-1 study of first-line therapy of MET-positive, HER2-negative gastric/EGJ cancer compared rilotumumab (15 mg/kg) plus ECX with placebo plus ECX was performed. OS (9.6 mo *vs* 11.5 mo, HR = 1.37, *P* = 0.021) and objective response rate (ORR: 30.0% *vs* 39.2%, OR = 0.67, *P* = 0.027) were significantly inferior in the rilotumumab group, and subgroup analysis also showed that no subgroups appeared to be benefit with rilotumumab arm regardless of the degree of MET positivity[63]. This result is contrary to that of a phase Ⅰ/Ⅱa study, which contained a larger number of Asian patients (18%) than the RILOMET-1 population (1%). The different racial distribution may have contributed to the opposite outcomes between the two studies. Thus, the phase Ⅲ RILOMET-2 study has been performed to investigate the efficacy of rilotumumab in combination with cisplatin/capecitabine regimen as the first line chemotherapy among Asian patients with unresectable gastric/EGJ cancer[64].

The METGastric study of onartuzumab (10 mg/kg) for the treatment of HER2-negative, MET-positive metastatic gastric or EGJ adenocarcinoma without prior treatment, used onartuzumab in combination with the 5-fluorouracil (5-FU)/leukovorin/oxaliplatin (FOLFOX) regimen and compared outcomes with FOLFOX alone[65]. The addition of onartuzumab to FOLFOX was ineffective in the intention-to-treat analysis and OS, PFS and ORR were not significantly different between the two groups. However, addition of onartuzumab showed a marginal effect in OS for the moderate-to-strong MET positive subgroup (9.7 mo *vs* 11.0 mo, HR = 0.64, *P* = 0.062). Grade 3 or 4 adverse events were more common in the onartuzumab arm. Furthermore, a French phase Ⅱ study that compared FOLFOX plus rilotumumab or panitumumab, an anti-EGFR antibody, with FOLFOX alone for first-line treatment of metastatic, HER2-negative gastric or EGJ adenocarcinoma showed that adding panitumumab or rilotumumab seemed more toxic and was not more effective than mFOLFOX6 alone[66]. Considering recent outcomes of phase Ⅱ/Ⅲ studies of rilotumumab and ornatuzumab, targeting HGF/c-MET in gastric cancer has little rationale for further evaluation. However, cMET still has potential for promising biomarkers considering that c-MET-positive gastric/EGJ cancers have strong association with shorter OS and poor prognosis[67], and future research needs to search for other significant predictive factors for response to anti-HGF/c-MET therapy.

Akt and mTOR inhibitors

A phase Ⅰ study evaluated the combinatory effect of MK-2206, a potent pan-Akt inhibitor, with trastuzumab for treatment of HER2-positive, refractory gastric carcinoma. The rational was that the PI3K/Akt pathway is a main downstream signaling pathway of HER2 and is closely related with trastuzumab resistance. Oral MKN-2206 was given either 135 mg every week or 60 mg every other day with trastuzumab 8 mg/kg intravenously on day 1 every 3 wk. Clinical benefit response rate including stable disease more than 4 mo was 24%, and median time to progression was 72 d[68].

The PI3K/Akt pathway might be successfully inhibited by targeting mTORC1 kinase, and the development of rapamycin analogs (*e.g*., everolimus, temsirolimus) have been promoted[15]. A multicenter phase Ⅱ study of everolimus, an oral inhibitor of mTOR, in patients with refractory metastatic gastric cancer showed a disease control rate of 56.0%, PFS of 2.7 mo (95%CI: 1.6-3.0 mo) and OS of 10.1 mo (95%CI: 6.5-12.1 mo), which warrant further phase Ⅲ evaluation[69]. However, results of the phase Ⅲ GRANITE-1 study comparing everolimus with best supportive care for previously treated advanced gastric cancer were disappointing, and researchers failed to demonstrated significant benefit in OS (5.4 mo *vs* 4.3 mo, HR = 0.90, *P* = 0.124); indeed, PFS was significantly increased in the everolimus arm (1.7 mo *vs* 1.4 mo, HR = 0.66, *P* < 0.001)[70]. A phase Ⅱ multicenter study of low dose everolimus (10 mg on days 1, 8 and 15) plus cisplatin and a weekly 24-h infusion of high-dose 5-FU and leucovorin (cisplatin 35 mg/m^2 intravenous infusion for 24 h on days 1 and 8, 5-FU 2000 mg/m^2 and leucovorin 300 mg/m^2 intravenous infusion for 24 h on days 1, 8 and 15) for treatment-naïve gastric cancer was conducted but failed to increase ORR as in a preplanned statistical assumption (52.5%)[71]. However, in one case everolimus was tried after failure of 1st and 2nd line chemotherapy for a young male metastatic gastric cancer patient with multiple liver metastases. A subsequent mutational analysis revealed a PIK3CA hotspot mutation and pS6 overexpression in the primary tumor. The patient achieved stable disease for 1 year and pS6 expression was nearly abolished after two cycles of everolimus treatment[72]. Furthermore, a phase Ⅱ study of everolimus for refractory metastatic gastric and EGJ adenocarcinoma showed that a subgroup with strong pS6 expression (≥ 2 + IHC staining) was significantly correlated with better PFS and disease control rate[73]. Therefore, subgroup analysis for finding of positive predictive biomarkers in patients treated with everolimus needs to be performed.

STAT3 inhibitors and effect of SHP1 inducers

Few agents capable of directly targeting STAT3 have been developed, and clinical trials of STAT3 inhibitors in the treatment of gastric cancer are lacking. A recent phase Ⅰ study reported that OPB-31121, an oral STAT3 inhibitor, showed an overall response rate

Table 2 Ongoing clinical trials of target therapy in advanced gastric and esophagogastric junction adenocarcinoma

Trial identifier	Line of treatment	Phase of study	Treatment arms	Primary endpoint
Akt/mTOR inhibitors				
NCT01613950[32]	Second/third	Ib	AUY922/BYL719	MTD
Immune checkpoints inhibitors				
NCT02335411 (KEYNOTE-059)[91]	Third	Ⅱ	Cohort 1: pembrolizumab monotherapy Cohort 2: pembrolizumab + 5-FU/cisplatin or capecitabine/cisplatin	ORR
NCT02370498 (KEYNOTE-061)[92]	Second	Ⅲ	Pembrolizumab *vs* paclitaxel	PFS, OS
Multikinase inhibitors				
NCT02015169[107]	Neoadjuvant	Ⅱ	XELOX + lapatinib	R0 resection rate
NCT01913639[108]	First	Ⅱ	FOLFOX + regorafenib	PFS

PFS: Progression free survival; OS: Overall survival; MTD: Maximum tolerated dose; 5-FU: 5-fluorouracin; ORR: Objective response rate; XELOX: Capecitabine/oxaliplatin; FOLFOX: 5-fluorouracil/leucovorin/oxaliplatin; mTOR: Mammalian target of rapamycin.

of 44.4% assessed as stable disease in advanced solid tumors including gastric cancer[74]. However, STAT3 not only up-regulates various target oncogenes associated with gastric carcinogenesis and metastasis, but is closely related with drug resistance of standard chemotherapeutic agents including 5-FU, cisplatin and adriamycin in gastric cancer[75-77]. In this regard, targeting of STAT3 remains a critical issue in the treatment of gastric cancer, and development of specific and effective inhibitors of STAT3 should be further investigated. Several natural compounds[76,78-80] and pharmacologic medicines, such as proton pump inhibitors[75,81], inhibit STAT3 activity in *in vitro* and *in vivo* studies of gastric cancer. These agents are expected to show a synergetic effect or enhance chemosensitivity when combined with standard chemotherapy agents.

Several natural compounds inhibit the STAT3 activation pathway through induction of SHP1 in hematopoietic cancer cell lines[42,43,82-85] and hepatoceullar carcinoma (HCC) cell lines[86,87]. We recently showed that plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), a quinonoid constituent extracted from the roots of the medical plant *Plumbago zeylanica* L, suppresses STAT3 activity and consequently targets gene expression *via* induction of SHP1 in gastric cancer cells[88]. Because most gastric cancer cells showed reduced or lack of expression of SHP1, a therapeutic strategy to indirectly inhibit STAT3 pathway might be an alternative option in pharmacologic treatment of gastric cancer and SHP1 may play pivotal roles in this signaling pathway. As mentioned above, several MKIs, such as sorafenib and sunitinib, show a significant link between SHP1 and suppression of STAT3 activity in HCC cells.

Immune checkpoint inhibitors: anti-PD-1/PD-L1 antibodies

The anti-CTLA-4 monoclonal antibody tremelimumab was developed and a phase Ⅱ study was performed to evaluate its use in second-line chemotherapy in advanced gastric and esophageal adenocarcinoma. However, the results were disappointing and only one patient achieved partial response among 18 enrolled patients, and stable disease was observed only in four patients[89]. Concerning the PD-1/PD-L1 pathway, pembrolizumab (MK-3475), an anti-PD-1 monoclonal antibody, and avelumab (MS0010718C), an anti-PD-L1 monoclonal antibody, have been developed. Pembrolizumab was tried for rescue therapy of recurrent or metastatic gastric or EGJ adenocarcinoma, which were positive for PD-L1, in the KEYNOTE-012 study[90]. ORR by central review was 22.2%, PFS 1.9 mo and OS 11.4 mo, and pembrolizumab showed durable efficacy and manageable safety profile for the heavily pre-treated, PD-L1 positive population. Further studies to support the efficacy of pembrolizumab in advanced gastric cancer are now in progress. For example, KEYNOTE-059 (NCT02335411) is a phase Ⅱ study of pembrolizumab monotherapy or in combination with standard chemotherapy[91] and KEYNOTE-061 (NCT02370498) is a phase Ⅲ study to compare pembrolizumab monotherapy with paclitaxel as the second-line therapy[92] (Table 2).

In Japan, avelumab was tried for refractory stage Ⅳ gastric and EGJ adenocarcinoma. A dose of 10 mg/kg was administered intravenously every 2 wk until progression. Most of adverse events were grade 1 or 2, ORR was 15.0% and PFS was 11.9 wk. Additional studies to evaluate the efficacy of avelumab and biomarkers from tumor tissue and blood samples including PD-L1 expression need to be evaluated[93].

MKIs

RTKs play crucial roles in the development of proliferation, differentiation, migration/invasion and apoptosis in gastric cancer. Currently, various inhibitors targeting the tyrosine kinase motif have been developed, and some display concurrent inhibitory effects of multiple tyrosine kinases. One of the first generation MKIs was sorafenib, which can inhibit BRAF, VEGF receptor (VEGFR) and platelet-derived growth factor receptor (PDGFR)[94]. A phase Ⅱ study investigated the efficacy of sorafenib in combination with docetaxel and cisplatin as the first-line chemotherapy in metastatic gastric or EGJ adenocarcinoma. Partial response was achieved in

41% (90%CI: 28%-54%), and the median PFS was 5.8 mo (90%CI: 5.4-7.4 mo) and median OS was 13.6 mo (90%CI: 8.6-16.1 mo). No additional toxicities were observed by adding sorafenib to docetaxel/cisplatin regimen. The results of this study warranted further evaluation of sorafenib in chemotherapy of gastric cancer[95]. However, another multicenter phase Ⅱ study of oxaliplatin and sorafenib as the second-line chemotherapy after failure of cisplatin/fluoropyrimidine regimen in advanced gastric adenocarcinoma revealed a median PFS of 3.0 mo (95%CI: 2.3-4.1 mo) and median OS of 6.5 mo (95%CI: 5.2-9.7 mo), which failed to support the implementation of a phase Ⅲ study[96]. Sorafenib was also evaluated for combination therapy with oral fluoropyrimidine and cisplatin, such as S-1/cisplatin[97] and capecitabine/cisplatin[98], in phase Ⅰ studies. Both studies showed tolerable safety profile and acceptable efficacy.

Lapatinib is a MKI that competitively inhibits ATP binding of tyrosine kinase in both HER2 and EGFR, and which is approved for the treatment of HER2-positive breast cancer[99]. Two large-scale, randomized, phase Ⅲ trials were recently reported. The researchers evaluated the efficacy and safety of lapatinib in HER2-positive, advanced or metastatic gastric and EGJ adenocarcinoma. The LOGiC study addressed lapatinib as the first-line chemotherapy in combination with capecitabine/oxaliplatin, and lapatinib arm was compared with capecitabine/oxaliplatin alone[100]. Median OS was not significant between both arms (12.2 mo *vs* 10.5 mo, P = 0.349), while PFS was significantly longer (6.0 mo *vs* 5.3 mo, P = 0.0381) and ORR was higher (53% *vs* 39%, P = 0.0031) in the lapatinib arm. Subgroup analysis for OS revealed that Asians and younger patients (< 60 years) showed significant benefit. The TyTAN study compared lapatinib plus paclitaxel with paclitaxel alone in the second-line treatment of gastric cancer in an Asian population[101]. This study showed no significant difference of median OS and PFS between both arms (11.0 mo *vs* 8.9 mo, P = 0.1044; 5.4 mo *vs* 4.4 mo, P = 0.2441; respectively). However, better efficacy was observed in the lapatinib arm in HER2-3+ subgroup. Further studies are warranted to examine the factors predicting good responders to lapatinib therapy.

Several novel MKIs have been investigated for the treatment of refractory gastric cancer. Findings were presented at the ASCO 2015 meeting. Among them, regorafenib, which inhibits multiple tyrosine kinases related to angiogenesis (VEGFR1-3), tumor microenvironment [PDGFR-β, fibroblast growth factor receptor (FGFR)] and oncogenesis (KIT), was previously developed and reported as effective in colon cancer and gastrointestinal stromal tumors (GISTs)[102]. The phase Ⅱ INTEGRATE study was designed and performed to investigate the efficacy of regorafenib in refractory, metastatic gastric and EGJ adenocarcinoma by comparing regorafenib 160 mg/d with placebo[103]. PFS was significantly increased in regrafenib group (2.6 mo *vs* 0.9 mo, HR = 0.40, P < 0.0001), however, OS was not significantly different between two groups (5.8 mo *vs* 4.5 mo, HR = 0.74, P = 0.11). An interesting thing is that HR = for PFS was significantly lower in Korean patients than in Western patients from Canada and Australia, which indicates that regorafenib might be more effective in Asian patients. Pazopanib is another potent MKI of VEGFR1-3, PDGFRα/β and FGFR1/3, and was previously approved by the United States Food and Drug Administration for the treatment of patients with advanced renal cell carcinoma[104]. A phase Ⅱ study was performed and reported the combinatory effect of pazopanib with capecitabine/oxaliplatin regimen as the first-line chemotherapy in metastatic gastric and EGJ cancer. ORR was 57.6% and adverse events of grade 3-4 were neutropenia (15.1%), anemia and thrombocytopenia (both 10.6%)[105].

CONCLUSION

Many studies have focused on revealing biologic relevant mechanism of development and progression of gastric cancer, and many medical agents targeting these pathways have been validated in clinical trials. However, most of them failed to reach significant benefits in phase Ⅲ trials, and novel therapeutic strategies are necessary in the future. To achieve this goal, individualized and precise target therapy should be planned on the basis of exploration of biologic characteristics of individual gastric cancer patients. In addition, targeting multiple RTKs rather than focusing on single pathway and attempts to overcome chemoresistance and enhance synergism with standard chemotherapeutic agents are expected to be prevalent. These approaches will hopefully lead to a more effective treatment, perhaps even conquest, of gastric cancer.

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REVIEW

Auto immune hepatitis

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Abstract

To provide an update of the latest trends in epidemiology, clinical course, diagnostics, complications and treatment of auto immune hepatitis (AIH). A search of the MEDLINE database was performed using the search terms: "auto immune hepatitis", "clinical presentation", "symptoms", "signs", "diagnosis", "auto antibodies", "laboratory values", "serology", "histopathology", "histology", "genetics", "*HLA* genes", "*non-HLA* genes", "environment", "epidemiology", "prevalence", "incidence", "demographics", "complications", "HCC", "PBC", "PSC", "corticosteroid", "therapy", "treatment", "alternative treatment". English-language full-text articles and abstracts were considered. Articles included reviews, meta-analysis, prospective retrospective studies. No publication date restrictions were applied. AIH is an immune meditated progressive inflammatory liver disease that predominantly affects middle-aged females but may affect people of all ages. The clinical spectrum of AIH is wide, ranging from absent or mild symptoms to fulminant hepatic failure. The aetiology of AIH is still unknown, but is believed to occur as the consequence of an aberrant immune response towards an un-known trigger in a genetically susceptible host. In the absence of a gold standard, diagnosis is based on the combination of clinical, biochemical and histopathological criteria. Immunosuppressive treatment has been the cornerstone of treatment since the earliest description of the disease in 1950 by Waldenström. Such treatment is often successful at inducing remission and generally leads to normal life expectancy. Nevertheless, there remain significant areas of unmet aetiological a clinical needs including fundamental insight in disease pathogenesis, optimal therapy, duration of treatment and treatment alternatives in those patients unresponsive to standard treatment regimens.

Key words: Auto immune hepatitis; Diagnosis; Liver; Epidemiology; Treatment

Core tip: Autoimmune hepatitis (AIH) is a chronic inflammatory liver disorder of unknown aetiology, which can lead to hepatic failure and premature

death when untreated. In AIH there is no existence of a pathognomonic feature and therefore the diagnosis rests on a combination of immunological, biochemical, and histological features together with exclusion of other liver diseases. Due to large heterogeneity of the disease, AIH might be unrecognised. Immunosuppressive treatment has been the cornerstone of treatment. Such treatment is often successful at inducing remission. For most patients life long treatment is indicated. In patients in whom all treatments fail, liver transplantation remains a final option.

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INTRODUCTION

The first to describe a chronic form of hepatitis in young women was Jan Waldenström in 1950[1]. Later, the disease was associated with other autoimmune diseases and was termed "lupoid hepatitis" because of the presence of antinuclear antibodies and lupus erythematosus cells[2]. These observations led to the idea that the foundation of this disease was a loss of immunological tolerance. The term Auto Immune Hepatitis (AIH) in its current meaning was introduced by Mackay and colleagues in 1965 when the concept of autoimmunity was acknowledged at an international meeting[3].

AIH is now recognized as a relatively rare chronic inflammatory liver disease predominantly affecting females in which a loss of tolerance against hepatic tissue is assumed. Based on the type of serum auto-antibodies, AIH can be subdivided into two types: type 1 AIH, identifiable by antinuclear antibodies (ANA) and/or anti-smooth muscle antibodies (SMA), and type 2 AIH, predominantly found in children and defined by antibodies against liver kidney microsomes type 1 (anti-LKM-1) or for anti-liver cytosol type 1 antibodies[4,5].

EPIDEMIOLOGY

There are few studies that have investigated the epidemiology of AIH. The majority of these studies are hampered by the fact that no predefined criteria for disease diagnosis were applied. In some older studies there has been admixture of patients with chronic hepatitis C and finally some of the studies may have been subject to tertiary referral bias (Table 1).

Nevertheless, incidence data are more or less comparable in Western Europe, ranging from 0.8 to 3 per 100000 with a prevalence ranging from 11 to 24 per 100000[5-9]. In Asia AIH seems to be less frequent, with incidence numbers ranging between 0.08 and 0.15 in Japan[10].

Substantially higher prevalence data of 42.9 cases per 100000 were found in a well defined native Alaskan population, although it should be noted that this study involved a small catchment area and a very limited number of patients[11]. Based on the available studies it is estimated that 11%-20% of all cases of chronic hepatitis in Western countries is caused by AIH[12]. The prevalence of AIH is still gradually increasing. Whether or not this reflects a true rise in incidence, as seen in other immune-mediated diseases like Crohn's disease, increased awareness of the disease or different diagnostic criteria is unknown.

Women are affected more frequently than men with a sex ratio of around 4:1[8]. In women a bimodal age pattern is usually seen, one in the late teens and one around the menopause but it should be stressed that disease can develop in all age groups and both genders[4,13].

PATHOGENESIS

The etiology of AIH remains unknown and fundamental questions regarding disease pathogenesis remain to be resolved. It is generally believed that AIH occurs in a genetically susceptible host as the consequence of an exaggerated immune reaction towards hepatic tissue[14]. Such a response can occur when effector lymphocyte responses are abundant and inappropriate leading to tissue damage, or, alternatively, when there is a numerical and/or functional defect in regulatory T cells (Treg) controlling such responses. This defect is more obvious at disease presentation than during treatment induced remission, where a partial recovery is observed.

Whilst abundant pro-inflammatory responses have been identified in most, if not all immune-mediated diseases, it has been very difficult to gain evidence for a primary defect in regulatory T cells in the majority of these diseases. Tregs isolated from children and adults with AIH were profoundly dysfunctional, suggesting that an underlying Treg deficiency plays a permissive role in the pathogenesis of AIH[15-17].

More recent studies omitted to find either functional or numerical Treg impairments in AIH patients and thus the question as to whether AIH is the result of defective immunoregulation warrants further investigation.

A third, not mutually exclusive mechanism may relate to molecular mimicry, which has been proposed as a mechanism by which exogenous substances may trigger an immune response against autoantigens. Such a response may spark an inflammatory reaction and the resulting hepatocellular injury may give rise to the release of other previously hidden antigens that may further fuel the inflammatory reaction. Exogenous pathogens implicated in this process include,

Table 1 Studies of incidence and prevalence of autoimmune hepatitis

Ref.	Year	Cases	Incidence/100000	Prevalence/100000
Toda *et al*[10]	1997	496	0.8	-
Whalley *et al*[125]	2007	200	3.0	-
Werner *et al*[9]	2008	473	0.85	10.7
Grønbæk *et al*[7]	2014	1721	1.68	23.9
Gerven *et al*[8]	2014	1313	1.1	18.3
Ngu *et al*[39]	2010	138	2.0	24.5
Delgado *et al*[126]	2013	100	0.67	11.0
Primo *et al*[127]	2004	13	1.37	11.61
Hurlburt *et al*[11]	2002	77	-	42.9

amongst others, the hepatitis C virus. A sequence homology between HCV polyprotein and cytochrome P4502D6 (CYP2D6) was previously reported, which was identified as anti-LKM-1 autoantibodies[18,19]. Indeed, anti-LKM-1 is seropositive in up to 10% of HCV patients. Other proposed triggers include other hepatotrophic viruses, as well as drug induced liver injury caused by antibiotics (including nitrofurantoin and minocycline), statins and anti-TNF agents[20-25].

GENETIC FACTORS

Genetic factors have long been implicated in disease pathogenesis yet systematic studies addressing the genetic epidemiology of AIH including familial occurrence, disease concordance in twins or ethnic differences in disease prevalence are lacking. Nevertheless, there are several observations that support a genetic basis for AIH. These include the association with other autoimmune diseases with a known genetic basis in up to a quarter of patients[8]. Additionally, associations with alleles of the Major Histocompatibility Complex (MHC) that encode the Human Leucocyte Antigens (HLA) were already described in the late seventies and confirmed and refined thereafter in numerous studies in different ethnic groups[26-28]. Such associations are found with most autoimmune diseases, most likely because they contribute to the specificity of immune reactions. HLA typing of patients with AIH reveals strong association with the HLA-DRB1 locus, with the haplotypes DRB1*0301 (HLA-DR3) and DRB1*0401 (HLA-DR4) as the main susceptibility factor in white Northern Europeans and North Americans[27,29-31]. Intriguingly there is evidence for substantial genetic heterogeneity in AIH with different MHC associations in different ethnic populations. Thus, in Japanese patients HLA-DRB1*0405 is the most important susceptibility allele[32,33] whereas primary associations with DRB1*0404 were found in Mexican patients[34].

The HLA alleles not only determine overall disease susceptibility but appear also to act as modifiers of the clinical phenotype. For instance, HLA-DR4 was found to be associated with female gender, less severe disease, more common autoimmune disease, and older age of onset[35-40].

Despite the fact that the MHC loci confer a 6 to 7 fold increased disease risk, these variants alone cannot explain the genetic predisposition for AIH. Genes outside the MHC have only been studied in candidate gene approaches involving limited numbers, making them prone to overestimation of significance. Most extensively studied is the cytotoxic T lymphocyte antigen-4 (*CTLA-4*) gene[41,42]. A recent study in the Netherlands involving a substantial number of patients however observed no significant differences in allele and genotype frequencies of the *CTLA-4* gene between AIH patients and controls[43].

More recently, genome-wide association studies have emerged as a powerful and unbiased approach for the identification of new genetic susceptibility loci in autoimmune diseases. Very recently this methodology was applied in a multicentre cohort of type 1 AIH patients. This study confirmed the involvement of the MHC region and identified *SH2B3* as the first genetic risk factor outside the MHC region. In addition, several other loci were identified supporting the thesis that AIH has a complex genetic basis[27].

CLINICAL FEATURES

The clinical manifestation of AIH can range from mild or severe symptoms to fulminant hepatic failure[44]. In all patients with liver disease AIH should be considered, so that that appropriate treatment can be instituted without delay. Up to 40 percent of patients presents with acute hepatitis, characterizes by right upper-quadrant abdominal pain, fatigue, jaundice and arthralgia[45]. However a fulminant manifestation or a long sub clinical course with only minimal increase of liver enzymes and non specific symptoms, such as arthralgia or fatigue, may be seen[12,46-49] (Table 2).

Clinical manifestations of AIH may vary among ethnic groups. Thus, non-Caucasian patients (the majority being from African-American descent) had more aggressive disease at initial presentation, lower reaction to immunosuppressive therapy, and worse outcomes when compared to Caucasian patients[44]. Higher rates of cirrhosis were found in Hispanic *vs* Caucasian patients, and a trend towards worse survival among Asians[50].

Other autoimmune diseases are common in up to

Table 2 Presentation and symptoms in auto immune hepatitis

Acute hepatitis	
Chronic hepatitis	
Hepatomegaly	
Splenomegaly	
Spider naevi	
Palmar erythema	
Non specific symptoms:	
Tiredness	
Fever	
Loss of appetite	
Upper abdominal pain	
Arthralgia	
Extrahepatic autoimmune disease (most common mentioned):	
Thyroiditis	10%-23%
Primary biliary cirrhosis	10%-20%
Diabetes	7%-9%
Primary sclerosing cholangitis	2%-8%
Rheumatoid arthritis	2%-5%
Celiac disease	1%-2%

40% of AIH patients. They included, among others thyroid disease, diabetes, inflammatory bowel disease and rheumatoid arthritis. A recent study demonstrates that celiac disease is more prevalent among AIH patients compared to the general population[51]. In addition AIH may have cholestatic features that can resemble primary sclerosing cholangitis (PSC) and primary biliary cirrhosis (PBC) and overlap with these diseases have been described in 10%-20% and 2%-8% of cases, respectively[9,14,52-55] (Table 2). So far, there have not been uniform definitions or diagnostic criteria for the overlap of AIH with PBC or PSC. It is still under debate as to whether these overlap syndromes represent variants of the main autoimmune liver diseases or hallmarks of a separate entity[56]. The presence of features of different diseases can occur simultaneously as well as sequentially in each form of overlap syndromes. AIH and PBC are the most frequently described autoimmune liver diseases. The pattern of abnormalities in laboratory tests can help determine the origin of the disease. In AIH a hepatic pattern is found, and a primarily cholestatic pattern in PBC; in addition, elevation of IgG is characteristic of AIH, an increase in IgM is commonly found in PBC patients.

Due to an absence of a well validated scoring system for the diagnosis of PBC-AIH overlap, the criteria developed by Chazouillères *et al*[57] are commonly applied.

In various reports AIH-PSC overlap syndrome has been described and is characterised by ANA and/or SMA seropositivity, hypergammaglobulinaemia and interface hepatitis - all features typical of "classical" AIH - in conjunction with cholestatic biochemical changes, frequently associated with inflammatory bowel disease, and histological evolution to fibrous obliterative cholangitis, ductopenia, portal tract oedema and/or bile stasis[58].

DIAGNOSIS

The diagnosis is based on the combination of clinical and laboratory features and histological changes after exclusion of other causes of hepatitis[59].

Laboratory abnormalities

AIH is suggested by a patient with elevated Alanine-aminotransferase (ALT) and Aspartate transaminase (AST) activity, raised Immunoglobulin G (IgG), high titres of circulating antibodies, negative serum tests and exclusion of toxic hepatitis. However not all these laboratory findings need to be present in an individual patient.

Elevation of serum IgG is a common finding in AIH[60], but normal IgG levels may be found in up to 30% of patients[61,62].

Auto antibodies are the hallmark of AIH and can constitute an important part of the diagnostic work up. The classic antibodies associated with AIH are Antinuclear antibodies (ANA), anti-smooth-muscle antibodies (ASMA) and Anti Liver kidney microsomal (LKM-1). About 70%-80% of AIH patients have significant titres ($\geqslant$ 1:40) of ANA or ASMA and overall 3%-4% have anti LKM-1, while up to 20% are seronegative for these antibodies[60].

ANA are the most commonly found auto antibodies in AIH, yet are rather non-specific since they can be found in a large variety of diseases as well as in healthy individuals[63]. ANA may be the only antibody present or may occur in conjunction with ASMA. ASMA are the second major class of antibodies which have proved useful in the diagnosis of AIH. Although less prevalent than ANA they are more specific[64].

Autoantibody detection not only supports in the diagnosis but also classifies between type 1 and type 2 AIH. Type 1 AIH is associated with the presence of ANA and/or SMA and type 2 with the presence of anti-LKM-1 and/or anti-liver cytosolic-1 (LC-1). In Northern Europe and North America type 2 AIH accounts for less than 10% of all patients[55].

Antibodies to soluble liver antigen (SLA) or liver pancreas antigen (LP) are found in 10%-30% of patients with AIH. These antibodies are specific for AIH and may proof useful in the diagnosis[65]. Antibodies to actin and atypical peripheral anti-neutrophilic cytoplasm are also commonly seen in type 1 AIH, however their applicability is limited due to lack in specificity[59].

Liver histology

A liver biopsy is usually necessary to confirm the diagnosis, provide histological assessment of disease severity and exclude other causes of hepatitis. There are no individual histological criteria that prove the diagnosis of AIH[66]. Interface hepatitis (or piecemeal necrose) is the histological hallmark of AIH and is a process of inflammatory infiltration and erosion of the hepatic parenchyma at the junction of the portal

Table 3 Simplified diagnostic criteria for auto immune hepatitis[75]

Variable	Cutoff	Points
ANA or ASMA	⩾ 1:40	1
ANA or ASMA	⩾ 1:80	
or LKM-1	⩾ 1:40	2
or SLA	Positive	
IgG	> Upper normal limit	1
	> 1.10 times upper normal limit	2
Liver histology (evidence of hepatitis is a necessary condition)	Compatible with AIH	1
	Typical AIH	2
Absence of viral hepatitis	Yes	2
		⩾ 6: probable AIH
		⩾ 7: definite AIH

ANA: Antinuclear antibodies; ASMA: Anti-smooth-muscle antibodies; LKM-1: Anti Liver kidney microsomal; IgG: Immunoglobulin G.

tract[67]. It is found in 84%-98% of patients[13,45,68], but can also be seen in patients with drug-induced and viral hepatitis[68]. The infiltrates consist of hepatic mesenchymal cells containing lymphocytes, plasma cells and histiocytes that typically accompany these cells. Patients presenting with chronic AIH typically have plasma cells infiltrated at the interface and throughout the lobule. Plasma cells are not invariability present and paucity of plasma cells does not therefore exclude a diagnosis of AIH. They may be absent in up to one third of the patients[68,69].

In a recent study, emperipolesis and rosette formation appear superior histological predictors of AIH when compared to the typical histological features of interface hepatitis and plasma cells[70].

Diagnosis scoring system

Because there is no golden standard for the diagnosis of AIH, diagnostic scoring systems have been established that support the diagnosis in most of patients. The IAIHG scoring system, originally published in 1993[71] and revised in 1999[60], was developed as a search tool to ensure comparability of study populations. Despite a high degree of sensitivity (100%) and specificity (90%)[72-74], these criteria have been proven impractical in the day to day clinical practice.

In 2008 the IAIHG produced a simplified system for the diagnosis of AIH which is less complex and enhances applicability in clinical practice[75]. This system is based on four variables: presence and level of anti bodies, IgG concentration, typical histological features and absence of viral markers (Table 3). Recently three studies report that the simplified scoring system performs with high specificity (97%-99%) and lower sensitivity (81%-88%) when compared to the original diagnostic criteria yet requires further prospective validation[72,76,77].

TREATMENT

Indication of treatment

The short and long term efficacious of immune suppression in patients with AIH has been described unequivocally. When left untreated, an estimated 40% of patients will die within six months of diagnosis[78]. When treated adequately, the 20-year survival rate for all treated patients exceeds 80%, and life expectancy is similar to that of age and sex matched normal subjects from the same geographical area[79].

Updated treatment guidelines have recently been emerged by the European Association for the Study of the Liver (EASL) in 2015, the British Society of Gastroenterology in 2011 and the American Association for the Study of Liver Diseases (AASLD) in 2010[4,80,81]. Patients with AST levels 10-fold the upper normal limit, or fivefold the upper normal limit in concurrence with IgG levels at least twice the upper normal limit, or histological features of bridging necrosis or multiacinar necrosis, should be offered immunosuppressive treatment because of clear survival benefit (Table 4). Patients not satisfying these criteria must be personalized and treatment should be based on clinical judgement[4].

Standard treatment

Current therapeutic strategies for AIH consist of an induction with prednisone and frequently include subsequent addition of azathioprine (AZA) as steroid-sparing maintenance therapy[80]. Prednisone is introduced at a dose of 1 mg/kg with a maximum of 60 mg/d in monotherapy or a maximum of 30 mg/d in combination treatment[4,12]. After AST and ALT normalize, prednisone alone can be reduced by 10 mg/wk until a dose of 20 mg.

Patients treated with combination therapy can reduce prednisone by 5 mg/wk until 15 mg. A slower reduction is advised after this point[4,82]. For maintenance treatment AZA can be used at a dose 1-2 mg/kg per day either alone or in combination with low dose prednisone[4,83]. A recent review based on available randomised controlled trials found that prednisone monotherapy and prednisone in combination wit AZA are both feasible induction therapies for AIH, while maintenance therapy prednisone and AZA and Monotherapy AZA are superior to prednisone monotherapy[84]. AIH patients

Table 4 Indication for treatment of auto immune hepatitis (adapted from Manns *et al*[4])

Absolute	Relative
Serum AST ⩾ 10 fold ULN	Symptoms (fatigue, arthralgia, jaundice)
Serum AST ⩾ 5 fold ULN and IgG level ⩾ twice normal	Serum AST and/or IgG less than absolute criteria
Bridging necrosis or multiacinar necrosis on histological examination	Interface hepatitis

AST: Aspartate transaminase; ULN: Upper limit normal; IgG: Immunoglobulin G.

treated with corticosteroids and/or AZA have the risk of many side effects on both drugs. The side effects of long term treatment with corticosteroids are well known; acne, moon shape face, striae, weight gain and loss of bone density. Adverse effects of thiopurines are common and generally occur shortly after the start of therapy. They include allergic reactions, flu-like illness, nausea fever, malaise, rash, abdominal pain, hepatotoxicity, pancreatitis and myelosuppression[83,85-87]. The principal side effects of AZA are cytopenia and liver test abnormalities, which may be difficult to distinguish from inherent AIH disease activity.

Remission and relapse

Remission of previously symptomatic patients is defined as a complete normalisation of all inflammatory parameters, including AST, ALT, bilirubine, IgG, recovery from symptoms and inactive liver histology[4,9,82]. In 80%-90% of patients with moderate/severe AIH, serum ALT decreases after starting treatment. Usually a decrease is seen within two weeks. As transaminase decrease, clinical symptoms revolve and liver functions shows marked improvement within 3-6 mo after starting prednisone treatment either with or without AZA[81].

There is no prescribed duration of the length of treatment. Because histological restore lags behind clinical and biochemical improvement by 3-8 mo, treatment should be continued for at least this period[88,89]. Proper patient selection including sustained remission on immunosuppressive Monotherapy for a minimum of 2 years can markedly improve the success rate of treatment withdrawal[90]. The AASLD and EASL guidelines recommend treatment withdrawal, when serum liver and immunoglobulin levels have been repeatedly normal for a period of at least two years. Liver biopsy prior to termination of treatment is preferred[4,80]. Relapse is characterized by an increase in ALT levels (three times upper normal limit) and/or increase of serum IgG level to more than 2 g/L following tapering of steroid doses or after complete withdrawal of immunosuppression[4]. Literature from the 1970s showed a high risk of relapse after drug withdrawal[88,91], but this was later disputed and it was recommended that drugs withdrawal should be attempted[92]. A more recent retrospective analysis found that relapse occurred in almost all patients with AIH when immunosuppressive medication was discontinued or tapered[4,92,93]. Relapse occurred despite prior attainment of complete remission, including a histological inactive follow up biopsy prior to tapering in a subgroup of patients. In patients who have relapsed once, a subsequent attempt to withdrawal therapy was invariably associated with the re-occurrence of a relapse[93]. Since repeated relapses were associated with a poorer long term prognosis patients should receive life long treatment[94,95]. A lifelong follow up should occur in patients who successfully stopped immunosuppression, while a relapse can occur 10 years later[93].

Alternative treatment

In up to 10% of AIH patients, the therapeutic strategy of prednisone and AZA is unsuccessful, due to intolerable side effects or lack of clinical response[4,81]. In patients who fail on standard therapy, alternative immunosuppressive treatments have been tried with encouraging results. Cyclosporine[96-98], tacrolimus[99,100], methotrexate[101], cyclophosphamide[102] and mycophenolate mofetil[103-105] have been tried, with varying degrees of success, as a replacement for AZA.

In a small recent study allopurinol was added to the AZA or mercaptopurine treatment in patients who fail treatment due to ineffectiveness or intolerance, due to skewed thiopurine metabolism. The combination of low dose thioprines and allopurinol proved an effective and well-tolerated alternative in the treatment of AIH. Larger and controlled studies are needed to confirm these outcomes[106]. As an alternative for prednisone, budesonide is receiving considerable attention.

In two recent studies in patients with noncirrhotic AIH oral budesonide, in combination with azathioprine, induces and maintains remission. This treatment causes fewer steroid-specific side effects[107,108]. Routine use is not currently recommended, while the trial duration is short and the fact that no follow up date were presented[81]. AZA is the prodrug of 6-mercaptopurin (6-MP) and is converted into 6-MP in a nonenzymatic manner before exhibiting its antiproliferative and immunosuppressive properties. In patients with ulcerative colitis and Crohn's disease 6-MP has a beneficial role in AZA-intolerant patients[109]. In patients with AIH and AZA intolerance, 6-MP seems to be an effective and well-tolerated second line treatment[110]. The use of 6-thioguanine 6-TG), an agent more directly leading to down-stream active metabolites of AZA, showed clinical improvement in

three AIH patients intolerant to AZA. A prospective evaluation of 6-TG as possible immunosuppressive drug in AIH patients is warranted[111].

COMPLICATIONS AND PROGNOSIS

Complications in AIH are comparable to those seen in other liver diseases and in rare cases AIH presents by the occurrence of hepatic encephalopathy[112,113].

Liver fibrosis is often presence at diagnosis and a subgroup of patients have already cirrhosis at presentation[4,68] suggesting that the disease has gone unrecognized for a significant period prior to diagnosis. When left untreated, an etsimated 40% of patients will die within 6 mo of diagnosis[88,91,114]. In some patients without proper treatment, AIH progresses to cirrhosis and eventually Hepatocellular carcinoma (HCC). The presence of cirrhosis at diagnosis or during treatment and the need for long-term immunosuppressive therapy have been observed as risk factors for malignant transformation[115]. In addition risk factors for HCC furthermore include male gender, advanced stage disease, portal hypertension as ascites and esophageal varices[116]. HCC occurs in 1%-9% of AIH patients[116-118], which is less frequently compared to patients with chronic viral hepatitis[119]. Imaging with ultrasonography or computed tomography should be conducted every 6-12 mo. In patients who develop liver failure, liver transplantation needs to be considered[48,120]. When AIH is indicated for transplantation, transplanted patients, practically compared to other chronic liver diseases, have an excellent 5 year survival of between 78%-91%[121-123]. The recurrence rate of AIH after initial successful transplantation is problematic and occurs in around 30% of patients[124].

CONCLUSION

AIH is a relatively rare disease of unknown aetiology. Many factors contribute to the diagnosis, which is characterized by a female predominance, histologically evidence of periportal hepatitis in the absence of viral markers, hypergammaglobulinaemia, the presence of auto antibodies in serum, plasmacellular infiltrates and an optimal response to steroids in most patients. In AIH there is no existence of a pathognomonic feature and therefore the diagnosis rests on a combination of immunological, biochemical, and histological features together with exclusion of other liver diseases. Due to large heterogeneity of the disease, AIH might be unrecognised. The clinical manifestation of AIH can range from mild or severe symptoms to fulminant hepatic failure. AIH generally responds to immunosuppressive treatment and treatment is required as soon as the diagnosis is made. For most patients lifelong treatment is indicated. In patients in whom all treatment attempts fail liver transplantation needs to be considered.

AIH remains a major diagnostic and therapeutic challenge. Growing insights into the clinical presentation of AIH highlights the importance of evaluation of the current diagnostic criteria, role of genetic and environmental factors, as well as the development of new treatment strategies.

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ORIGINAL ARTICLE

Basic Study

Altered tryptophan hydroxylase 2 expression in enteric serotonergic nerves in Hirschsprung's-associated enterocolitis

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Informed consent statement: Informed written consent was obtained from the parents/legal guardians of all children enrolled in this study.

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Abstract

AIM: To determine if expression of colonic tryptophan hydroxylase-2 (TPH2), a surrogate marker of neuronal 5-hydroxytryptamine, is altered in Hirschsprung's-associated enterocolitis.

METHODS: Entire resected colonic specimens were collected at the time of pull-through operation in children with Hirschsprung's disease (HSCR, n = 12). Five of these patients had a history of pre-operative Hirschsprung's-associated enterocolitis (HAEC). Controls were collected at colostomy closure in children with anorectal malformation (n = 10). The distribution of expression of TPH2 was evaluated using immunofluorescence and confocal microscopy. Protein expression of TPH2 was quantified using western blot analysis in the deep smooth muscle layers.

RESULTS: TPH2 was co-expressed in nitrergic and cholinergic ganglia in the myenteric and submucosal plexuses in ganglionic colon in HSCR and healthy controls. Co-expression was also seen in submucosal interstitial cells of Cajal and PDGFRα^+ cells. The density of TPH2 immuno-positive fibers decreased incrementally from ganglionic bowel to transition zone

bowel to aganglionic bowel in the myenteric plexus. Expression of TPH2 was reduced in ganglionic bowel in those affected by pre-operative HAEC compared to those without HAEC and healthy controls. However, expression of TPH2 was similar or high compared to controls in the colons of children who had undergone diverting colostomy for medically refractory HAEC.

CONCLUSION: Altered TPH2 expression in colonic serotonergic nerves of patients with HSCR complicated by HAEC may contribute to intestinal secretory and motor disturbances, including recurrent HAEC.

Key words: Serotonin; Tryptophan hydroxylase 2; Hirschsprung; Enterocolitis; Ganglionic

Core tip: Despite optimal surgery, children with a history of Hirschsprung's disease (HSCR) complicated by Hirschsprung's-associated enterocolitis (HAEC) are at higher risk of long-term colonic dysfunction. Tryptophan hydroxylase-2 (TPH2) is a surrogate marker for neuronal 5-hydroxytryptamine (5-HT). We hypothesized that expression of TPH2 is altered in the colon of children with HAEC. We found the density of serotonergic nerves to be differentially reduced in the ganglionic colon of children with HSCR and HAEC compared to those without HAEC compared to controls, although expression is normalized in those with diverting colostomy. Abnormal neuronal 5-HT expression may contribute to post-operative colonic dysfunction in HSCR.

Coyle D, Murphy JM, Doyle B, O'Donnell AM, Gillick J, Puri P. Altered tryptophan hydroxylase 2 expression in enteric serotonergic nerves in Hirschsprung's-associated enterocolitis. *World J Gastroenterol* 2016; 22(19): 4662-4672 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i19/4662.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i19.4662

INTRODUCTION

Hirschsprung's-associated enterocolitis (HAEC) is the most serious complication of Hirschsprung's disease (HSCR) and is the leading cause of disease-related mortality. It occurs in 17%-50% of patients with HSCR and may occur before or after a pull-through operation[1,2]. Although a scoring system for HAEC exists, there is no agreed definition of this condition. It is typically described as an inflammatory disease of the colon leading to a spectrum of symptoms ranging from abdominal distension and loose stools to life-threatening toxic megacolon[3,4]. The etiology and pathogenesis of HAEC are still incompletely understood. It has been proposed that intestinal barrier dysfunction, abnormal innate immunity and the presence of a disturbed microbiome are all potential contributors to its etiology[1]. However, given that the primary abnormality in HSCR is the absence of enteric ganglia in the distal colon, it follows that the enteric nervous system may have a role in the pathogenesis.

5-Hydroxytryptamine (5-HT), also commonly known as serotonin, is a major neuroendocrine signaling molecule. While the gut is the single largest reservoir of 5-HT, the wide range of its functions therein have only been elucidated relatively recently. The majority of enteric serotonin is stored in the mucosa in the enterochromaffin (EC) cells. Approximately 1%-5% of enteric serotonin is stored in the serotonergic enteric nerves, where it acts as a neurotransmitter[5,6]. The rate limiting step in the synthesis of 5-HT is the conversion of L-tryptophan to 5-hydroxytryptophan by tryptophan hydroxylase (TPH). The conversion of 5-hydroxytryptophan to 5-HT then occurs rapidly through the actions of L-amino acid decarboxylase[7]. However, the synthesis pathway of enteric 5-HT differs depending on whether 5-HT is being synthesized in EC cells, where the TPH1 isozyme of TPH predominates, or in serotonergic neurons, where TPH2 predominates. In this way, it is possible to indirectly evaluate the expression of neuronal 5-HT separately from that produced in EC cells[5,7].

5-HT has many roles, including activation of intrinsic reflexes such as peristalsis, vasodilation, and secretion. When released from EC cells, mucosal 5-HT has been demonstrated to promote inflammation - an activity which is counterbalanced by its re-uptake *via* the serotonin transporter (SERT)[5,8]. Abnormal mucosal 5-HT activity has been demonstrated in inflammatory and functional bowel disorders such as ulcerative colitis and irritable bowel syndrome[9]. Conversely, enteric neuronal 5-HT is anti-inflammatory and neuroprotective, an activity which has obvious importance in the setting of inflammation and enterocolitis, as neuronal damage can frequently result[8]. It has previously been reported that populations of mucosal EC cells are deficient in the ganglionic bowel of children with HSCR who have previously had HAEC. It is unclear if this is a facilitator or an effect of HAEC[10].

We hypothesized that, in children who had previously been treated for HAEC, neuronal 5-HT expression is altered compared to those who did not require treatment for HAEC. In this study we aimed to investigate the distribution of 5-HT in the aganglionic and ganglionic colon of children with HSCR and in healthy controls and to quantify expression of TPH2 in serotonergic enteric nerves of these patients.

MATERIALS AND METHODS

Specimen collection

The study was approved by the ethics committees of both centers (Our Lady's Children's Hospital Ethics Committee, GEN292.12; Temple Street Children's

University Hospital Research and Ethics Committee, 13.003). Informed written consent was obtained from parents/legal guardians prior to specimen collection. The procedures carried out during the study were in conformance with the principles expressed in the Declaration of Helsinki.

Full-length colonic specimens resected during pull-through operations for HSCR were obtained fresh from 12 patients, incorporating aganglionic, transition zone and ganglionic bowel (age range 3 mo-14 mo). Colonic control specimens were similarly obtained from the proximal colostomy limb of 10 patients at the time of descending/sigmoid colostomy closure in children following surgical correction of anorectal malformation (age range 7 mo-21 mo). The level of the most proximal extent of the transition zone was routinely confirmed by 3,3'-diaminobenzidine (DAB) immunohistochemistry probing for protein gene product 9.5 (PGP 9.5), which stains nerve cells. All experiments incorporated comparison of ganglionic bowel in HSCR with transition zone and aganglionic bowel as well as healthy controls.

Double-label immunofluorescence

Colonic sections were embedded in OCT compound [VWR, Ireland (361603E)] and snap frozen in liquid nitrogen. Twenty micron sections were cut and were fixed in 10% neutral buffered formalin (Sigma-Aldrich, Ireland [HT501128-4L]). Cell membranes were permeabilized by rinsing in 1% w/v PBS with 1% Triton X-100. Sections were blocked in 10% bovine serum albumin [BSA, Sigma-Aldrich, Ireland (A2153-50G)] diluted in 1% w/v PBS with 0.05% Tween® [Sigma-Aldrich, Ireland (P1379)] (PBST) for 90 min at room temperature to prevent non-specific antibody binding. Samples were incubated simultaneously in both primary antibodies of interest, diluted in 10% BSA, at 4 ℃ overnight. Antibodies to the following antigens were used to label specific cell types in the colonic wall: HuD (PGP 9.5) was used to label nerve cells; TMEM16A [anoctamin-1 (ANO1)])was used to label interstitial cells of Cajal (ICCs); platelet derived growth factor receptor-α (PDGFRα) was used to label PDGFRα$^+$ cells, neuronal nitric oxide synthase (nNOS) was used to label nitrergic neurons, vasoactive intestinal peptide (VIP) was used to label peptidergic neurons, and choline acetyltransferase was used to label cholinergic neurons. A detailed description of the primary antibodies used in the study is seen in Table 1. Following incubation in primary antibody solution, samples were rinsed intensively in 1% PBST, following which they were incubated in a solution containing both secondary antibodies specific to the host species of each primary antibody (Table 1), diluted in 10% BSA, for 90 min at room temperature. After intensive rinsing in 1% PBST, samples were counterstained with 4',6-diamidino-2-phenylindole (DAPI) nuclear counterstain [Thermo Scientific, Ireland (EN62248)]. Sections were mounted with glass coverslips using Mowiol® 4-88 fluorescence mounting medium [Sigma Aldrich, Ireland (81381-50G)], which was constituted according to manufacturer's specifications. Specimens were visualized using laser scanning confocal microscopy (LSM700 Confocal Microscope, Carl Zeiss MicroImaging GmbH, Jena, Germany). Resulting images were processed, including calculation of TPH2 immuno-positive cell counts, using ImageJ - an open-access software available from http://imagej.nih.gov/ij/.

Protein extraction and Western blot analysis

The mucosa was dissected from the deep smooth muscle layers at the time of specimen collection. Protein was extracted from the tunica muscularis layers to limit the probability of unwanted antibody binding to tryptophan hydroxylase 1 (TPH1), of which the mucosa is a substantial reservoir. Bowel tissue fragments were homogenised using a tissue homogeniser in radioimmunoprecipitation (RIPA) buffer containing 1% protease inhibitor cocktail [Sigma Aldrich, Ireland (P2714)]. Soluble and insoluble fractions were then separated by centrifugation at 4 ℃ at 3000 *g* over 30 min. The concentration of the supernatant was determined by means of a Bradford assay [Sigma-Aldrich Ltd., Arklow, Ireland (B6916)] using a standard curve generated from known concentrations of BSA. Novex® Bolt® LDS sample buffer and reducing agent [Biosciences, Dublin, Ireland (B0007 and B0009 respectively)] were added to each protein aliquot according to manufacturer's protocols. The protein concentration of each sample was then equilibrated with the addition of deionised water. Samples were denatured at 70 ℃ for 10 min and were then loaded onto an SDS polyacrylamide gel [Bolt® Novex 4%-12% Bis-Tris gel: Biosciences, Dublin, Ireland (NW04120BOX)] in NuPAGE® MES SDS running buffer [Biosciences, Dublin, Ireland (NP0002)] and separated by electrophoresis at 150 V. Proteins were then transferred from the gel to a 0.45 μm PVDF membrane at 30 V for 90 min.

Membranes were blocked in 3% dried skimmed milk dissolved in 1% PBST for 1 h to prevent non-specific antibody binding and were then incubated in primary antibody (Table 1) diluted in 10% BSA overnight at 4 ℃. Membranes were rinsed in 1% PBST for 4 h and were then incubated in species-specific secondary antibody (Table 1) for 90 min at room temperature. Following further rinses with 1% PBST for a minimum of 1 h, membranes were incubated in chemiluminescent substrate [SuperSignal™ West Pico Chemiluminescent Substrate, Thermo-Fischer, Ireland (34079)] for 5 min at room temperature before transfer to a chemiluminescence cassette for blot visualization. Protein expression levels were semi-quantitatively evaluated by densitometric analysis using the open-access image processing software

Table 1 Details pertaining to primary and secondary antibodies used in immunofluorescence and Western blot

Host species	Monoclonal/Polyclonal	Antigen	Product code	Manufacturer	Dilution
Rabbit	Polyclonal	Tryptophan hydroxylase 2	NB-100-74555	Novus biologicals (Cambridge, United Kingdom)	1:100
Mouse	Monoclonal	TMEM-16A (ANO1)	Ab190721	Abcam (Cambridge, United Kingdom)	1:200
Mouse	Monoclonal	HuD	sc-48421	Santa-Cruz Biotechnologies (Heidelberg, Germany)	1:100
Mouse	Monoclonal	Platelet-derived growth factor alpha	sc-21789	Santa-Cruz Biotechnologies (Heidelberg, Germany)	1:100
Goat	Polyclonal	Neuronal nitric oxide synthase	ab1376	Abcam (Cambridge, United Kingdom)	1:300
Mouse	Monoclonal	Vasoactive intestinal peptide	sc-25347	Santa-Cruz Biotechnologies (Heidelberg, Germany)	1:100
Mouse	Monoclonal	Choline acetyltransferase	ab49382	Abcam (Cambridge, United Kingdom)	1:100
Mouse	Monoclonal	GAPDH	ab9484	Abcam (Cambridge, United Kingdom)	1:2000
Rabbit	Polyclonal	Mouse (secondary)	ab6728	Abcam (Cambridge, United Kingdom)	1:10000
Donkey	Polyclonal	Rabbit (secondary)	ab6802	Abcam (Cambridge, United Kingdom)	1:10000
Donkey	Polyclonal	Rabbit (Alexa Fluor® 488)	ab150073	Abcam (Cambridge, United Kingdom)	1:500
Goat	Polyclonal	Mouse (Alexa Fluor® 594)	ab150116	Abcam (Cambridge, United Kingdom)	1:500
Donkey	Polyclonal	Goat (Alexa Fluor® 555)	ab150134	Abcam (Cambridge, United Kingdom)	1:500

Table 2 Clinical details of patients with Hirschsprung's disease included in the study

Characteristic	*n* = 12	
Gender	Male (*n* = 11)	Female (*n* = 1)
Median age at pull-through operation	5 mo	3-14 mo
Associated syndromes	Trisomy 21	*n* = 5 (2 with HAEC)
Pre-operative HAEC	Yes (*n* = 5)	No (*n* = 7)
Diverting/levelling stoma	Yes (*n* = 3)	No (*n* = 9)

HAEC: Hirschsprung's-associated enterocolitis.

ImageJ. Statistical analysis was performed using a statistical software package (SPSS v20.0). Non-parametric analysis including Mann-Whitney *U*-test was utilized in testing for differences in TPH2 protein expression between ganglionic and aganglionic colon in HSCR and healthy controls.

RESULTS

Double-labelled immunofluorescence

Basic clinical details regarding patients whose specimens were used in this study can be seen in Table 2. The specificity of our antibody to detect neuronal TPH2 was confirmed by the presence of TPH2 co-expression with HuD, a specific marker of nerve cell bodies, seen in the myenteric plexus of normally ganglionated bowel in HSCR and in healthy controls, while no evidence of expression of either was seen in the myenteric plexus of aganglionic bowel in HSCR (Figure 1A and B). There was a dense network of ANO1-immuno-positive ICC fibers seen in the myenteric plexus. There was no co-expression of TPH2 with ANO1 (Figure 1C), although the processes of ICCs did form dense network around ganglia immuno-positive for TPH2. Of interest, there was partial co-expression of TPH2 with PDGFRα in the cell bodies of PDGFRα$^+$ fibroblast-like cells in the myenteric plexus (Figure 1D).

In the submucosa of aganglionic bowel, HuD-positive nerve cell bodies were absent, although TPH2-positive fibers were still present in reduced density (Figure 2A). Confirmation of the presence of TPH2-immuno-positive nerve cell bodies in the submucosa of ganglionated bowel is seen in Figure 2B. TPH2 was co-expressed with ANO1 in the cell processes of submucosal ICCs (Figure 2C) and with PDGFRα in the cell bodies of PDGFRα$^+$ cells (Figure 2D).

The density of TPH2-immuno-positive cells reduced incrementally from colonic controls (Figure 3A) to ganglionic bowel in HSCR (Figure 3B), transition zone (Figure 3C) and aganglionic bowel (Figure 3D), where expression of TPH2 appeared markedly reduced in the myenteric and submucosal plexuses (Figure 3E). TPH2 was found to be co-expressed with ChAT-positive cholinergic nerve cell bodies in the myenteric plexus (Figure 4A), as well as with nNOS in nitrergic ganglia (Figure 4B). VIP-immuno-positive neurons did not co-express TPH2 in the myenteric plexus (Figure 4C) or in the submucosa (data not shown). Cholinergic nerve fibers in the circular muscle co-expressed TPH2 (Figure 4D).

Western blot analysis

A specific band was detected at approximately 60 kDa, consistent with the molecular weight of TPH2. No doublet bands were observed, confirming specific antibody binding to the TPH2 isozyme, as occurred when Western blot analysis was performed using whole tissue protein extract, which contains significant quantities of TPH1. In patients who had HAEC, TPH2 expression was reduced in the aganglionic bowel, transition zone and ganglionic bowel in HSCR compared to controls ($P = 0.044$) while there was a trend towards lower expression of TPH2 patients with HSCR who had never been treated for HAEC ($n = 7$) in aganglionic bowel compared to healthy controls ($P = 0.056$) (Figure

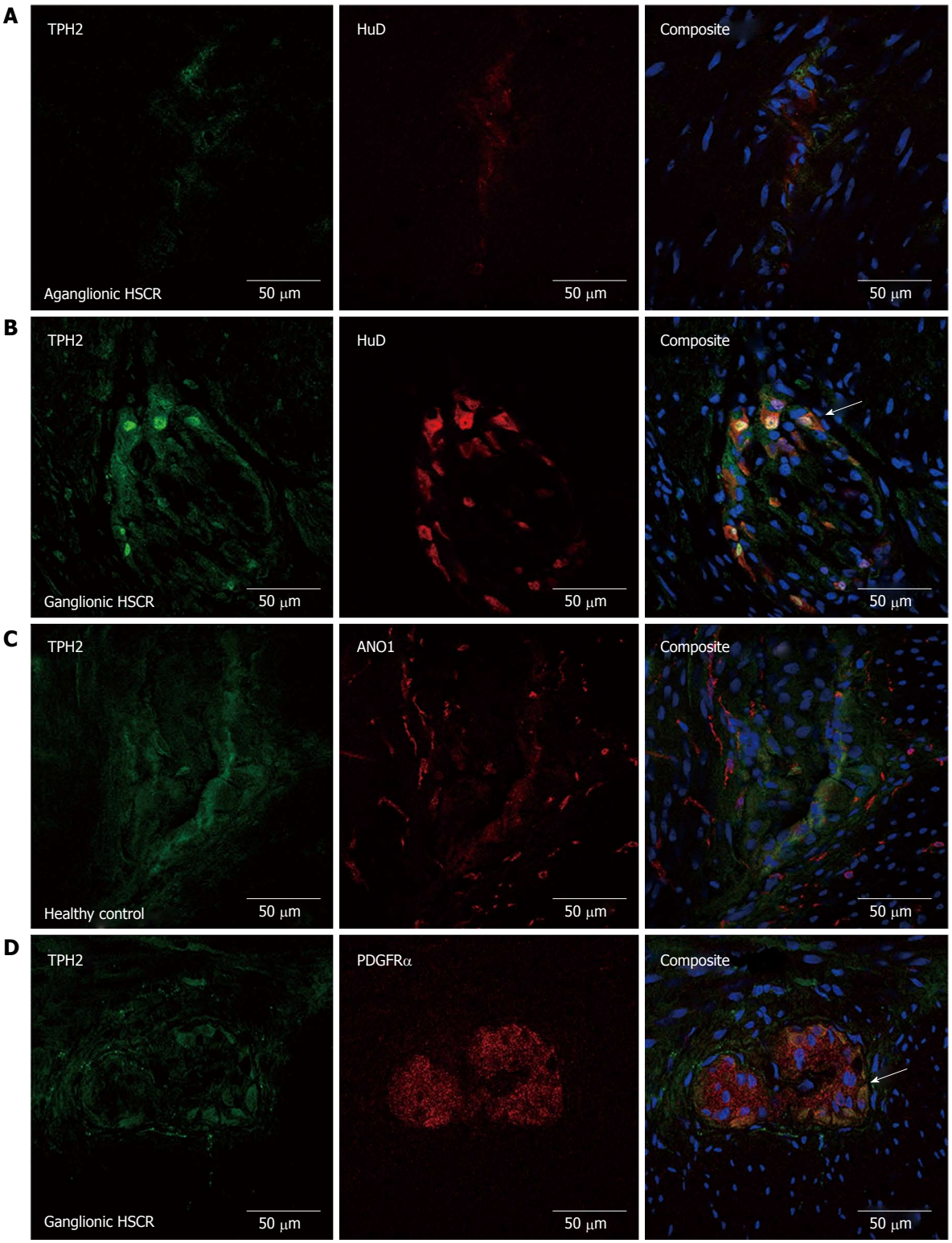

Figure 1 Confocal micrograph series demonstrating pattern of tryptophan hydroxylase-2 expression in the myenteric plexus of the colon. There is almost no HuD or TPH2 immunofluorescence seen in aganglionic colon in HSCR in (A), while there is clear co-expression (arrow head) in the nerve cell bodies in (B). While TPH2 and anoctamin-1 (ANO1) are not co-expressed in (C), the interstitial cells of Cajal (ICC) fibers form a dense network around TPH2-immuno-positive ganglia. TPH2 was co-expressed with PDGFRα cell bodies in the myenteric plexus, seen in (D). TPH2: Tryptophan hydroxylase-2; HSCR: Hirschsprung's disease.

5A). However, consistent marked recovery of TPH2 expression levels was noted in the colon of patients with HSCR, who had been treated for HAEC, but who had undergone formation of a diverting stoma due to failure of medical management (P = 0.002, n = 3). Such was the recovery that expression levels were even higher than in patients who had HSCR but who did not have a history of pre-operative HAEC (Figure 5B).

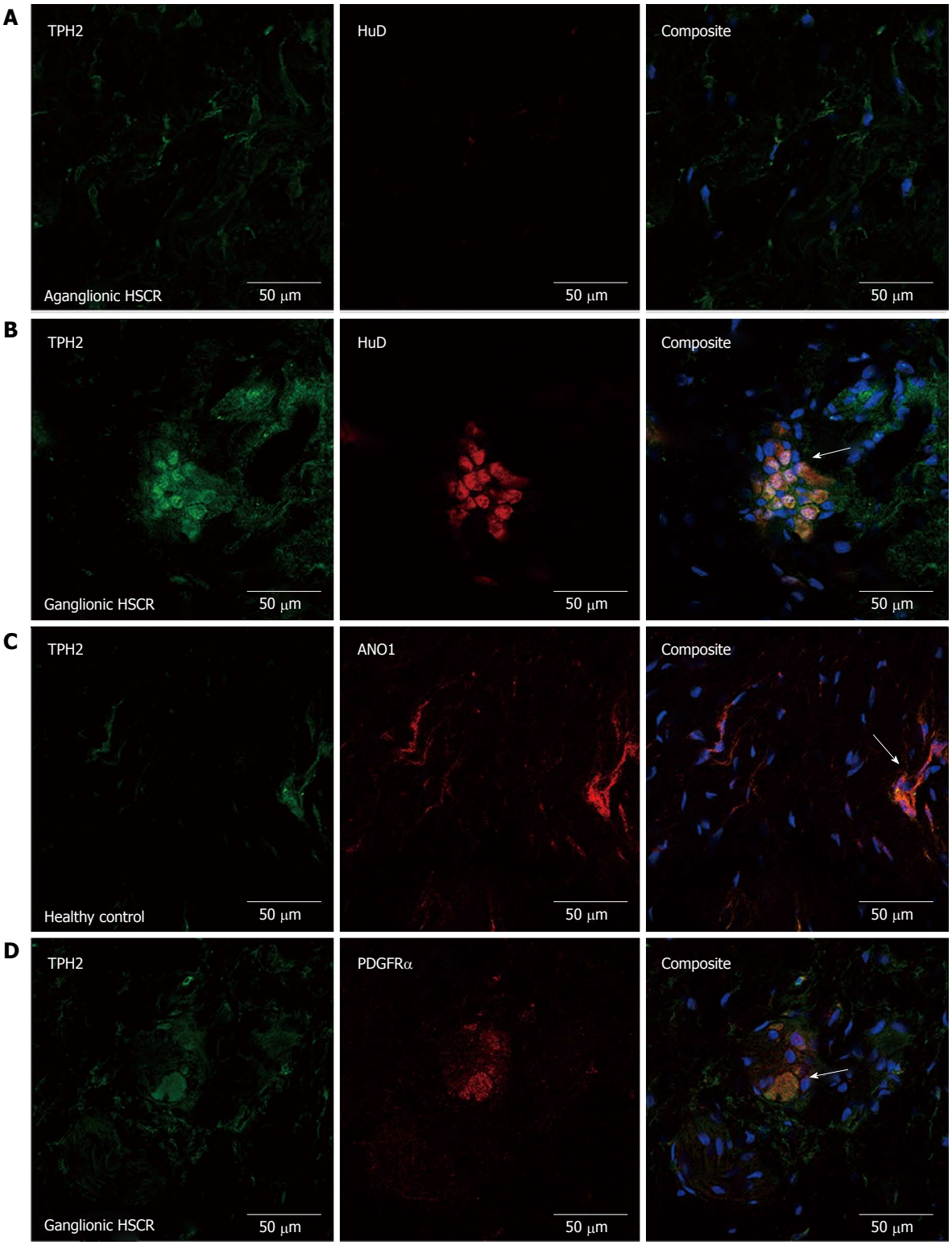

Figure 2 Confocal micrograph series demonstrating tryptophan hydroxylase-2 expression pattern in the submucosal plexus. Again, no HuD expression is seen in the aganglionic bowel, although there are some TPH2 immuno-positive fibres seen in (A). In ganglionic bowel in HSCR (B), TPH2 co-expressed with HuD (white arrow) in ganglion cells. There was co-expression of TPH2 with anoctamin (ANO)-positive submucosal interstitial cells of Cajals (ICCs) and PDGFRα^+ cells, seen in (C) and (D) respectively (white arrow). TPH2: Tryptophan hydroxylase-2; HSCR: Hirschsprung's disease.

DISCUSSION

Enterocolitis is reported as the presenting feature of HSCR in between 12.5% and 40.5% of cases, with many of these cases occurring in the neonatal period[2,11]. The histopathological changes seen in HAEC range from cryptitis to mucosal ulceration, transmural necrosis and colonic perforation[1]. Only recently has an understanding of the processes contributing to the pathogenesis of enterocolitis been developed. In the

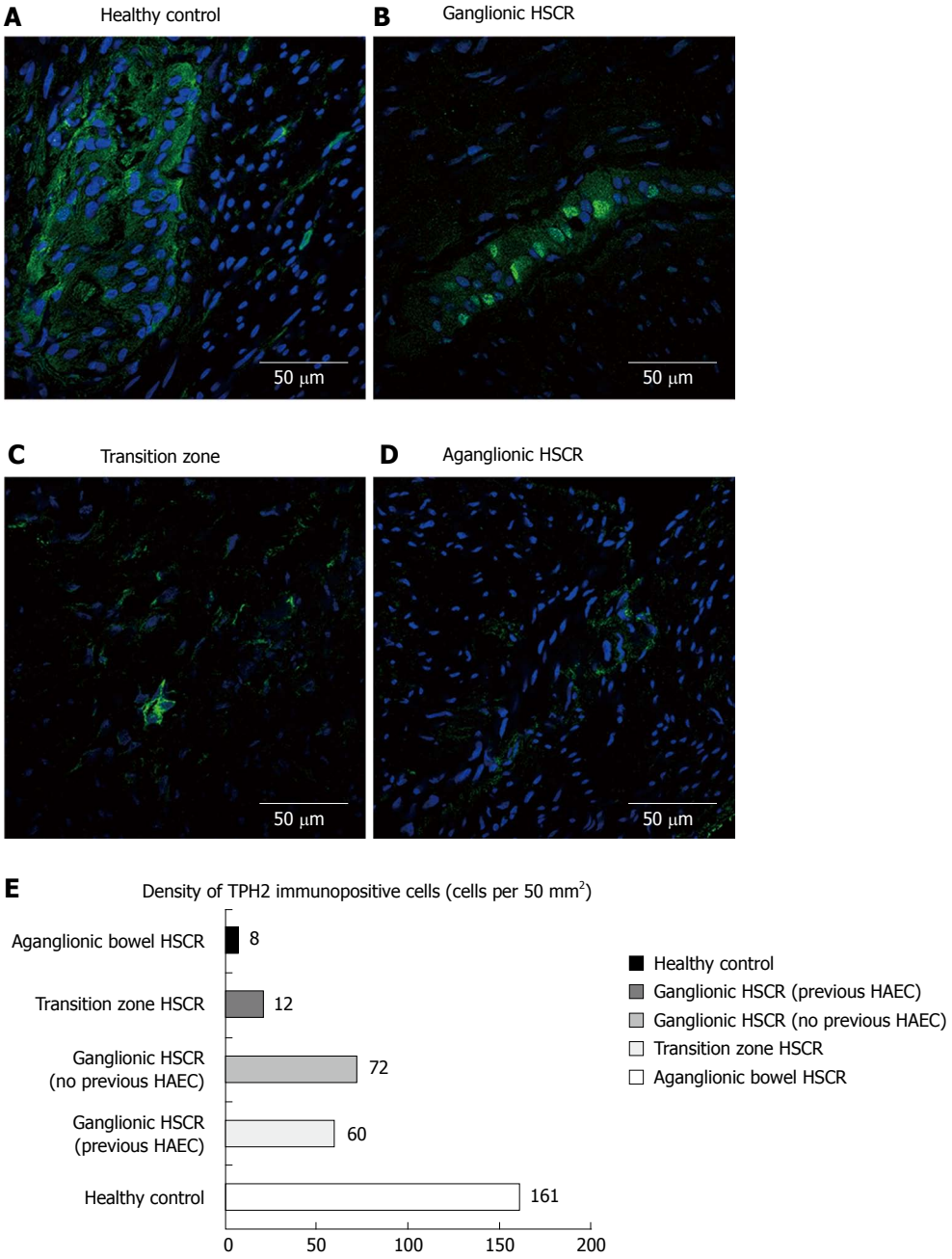

Figure 3 Double-labelled immunofluorescence. Series (A) to (D) demonstrate the incremental reduction in tryptophan hydroxylase-2 immuno-positive cells in the myenteric plexus from healthy control, to ganglionic bowel in HSCR through to aganglionic bowel. The bar graph in (E) demonstrates the mean cell counts of TPH2 immuno-positive cells, with a reduction seen particularly in transition zone and aganglionic bowel, and slight variation seen in the ganglionic bowel of those with pre-operative HAEC *vs* those who did not. TPH2: Tryptophan hydroxylase-2; HSCR: Hirschsprung's disease; HAEC: Hirschsprung's-associated enterocolitis.

colon, the enteric nervous system (ENS) is arranged into two plexuses: a submucosal plexus and a plexus that lies between the two deep smooth muscle layers, known as the myenteric plexus. The role of the myenteric plexus primarily concerns intestinal motility while the submucosal plexus regulates a myriad of epithelial functions such as enteric blood flow, immunity, release of enteric peptides from enteroendocrine cells, and epithelial transport[1,12]. It follows that the absence of a functioning ENS in the colon of those with HSCR will lead to dysregulation of these processes and, consequently, enterocolitis.

Although only 5% of 5-HT in the colon is neuronal in origin, it is notable that, in TPH1 knockout mice, gastrointestinal motility is preserved, even with stripping of the mucosa, indicating that only neuronal 5-HT is involved in gastrointestinal motility[5]. Additionally, animal models have demonstrated that neuronal 5-HT acts as a growth factor during enteric neurogenesis, promoting the development and survival of dopaminergic and GABAergic neurons[5,13]. It is thought that serotonergic neurons are large descending interneurons which, as well as being calbindin-positive, are cholinergic[14]. Our

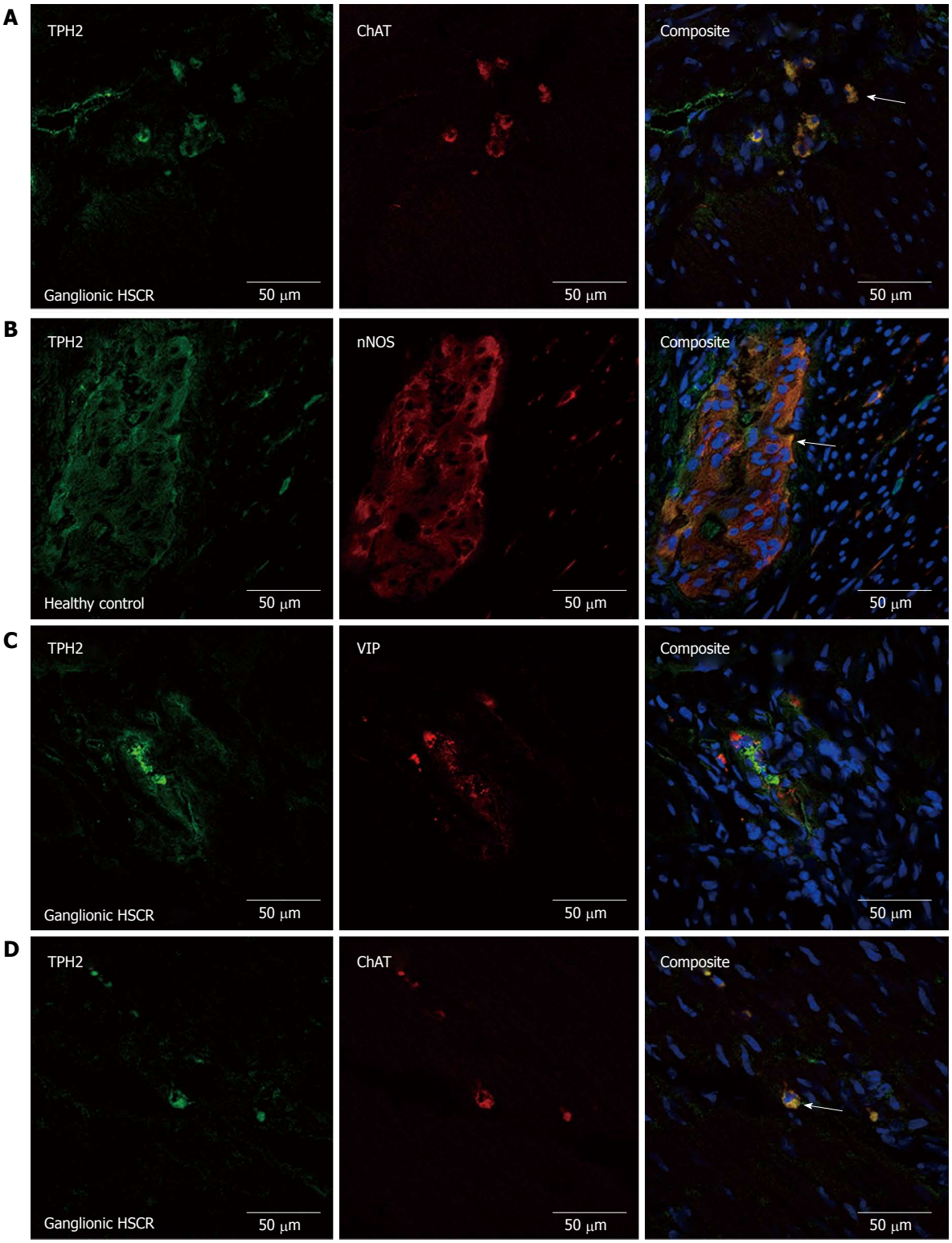

Figure 4 Confocal micrograph series. Confocal micrograph series demonstrating the cholinergic nature of TPH2 immuno-positive neurons in the myenteric plexus (A), with ChAT-TPH2 co-expression (white arrow). There was also co-expression of nNOS and TPH2 in the myenteric plexus (B) (white arrow) and submucosal plexus (not shown). VIPergic neurons did not express TPH2 in the myenteric plexus (C). Image (D) shows ChAT immune-positive cholinergic nerve fibres in the circular muscle layer, co-expressing TPH2. TPH2: Tryptophan hydroxylase-2.

findings concur with this observation, as we observed consistent co-expression of TPH2 with ChAT, a key enzyme in acetyl choline synthesis, in circular muscle nerve fibers.

Serotonergic nerves have previously been shown to make extensive synapses with myenteric ICCs, as well as nitrergic neurons. This indicates a functional role for serotonergic neurons in modulating nitrergic neurotransmission and pacemaker activity. Okamoto *et al*[15] has also observed that most colonic nitrergic

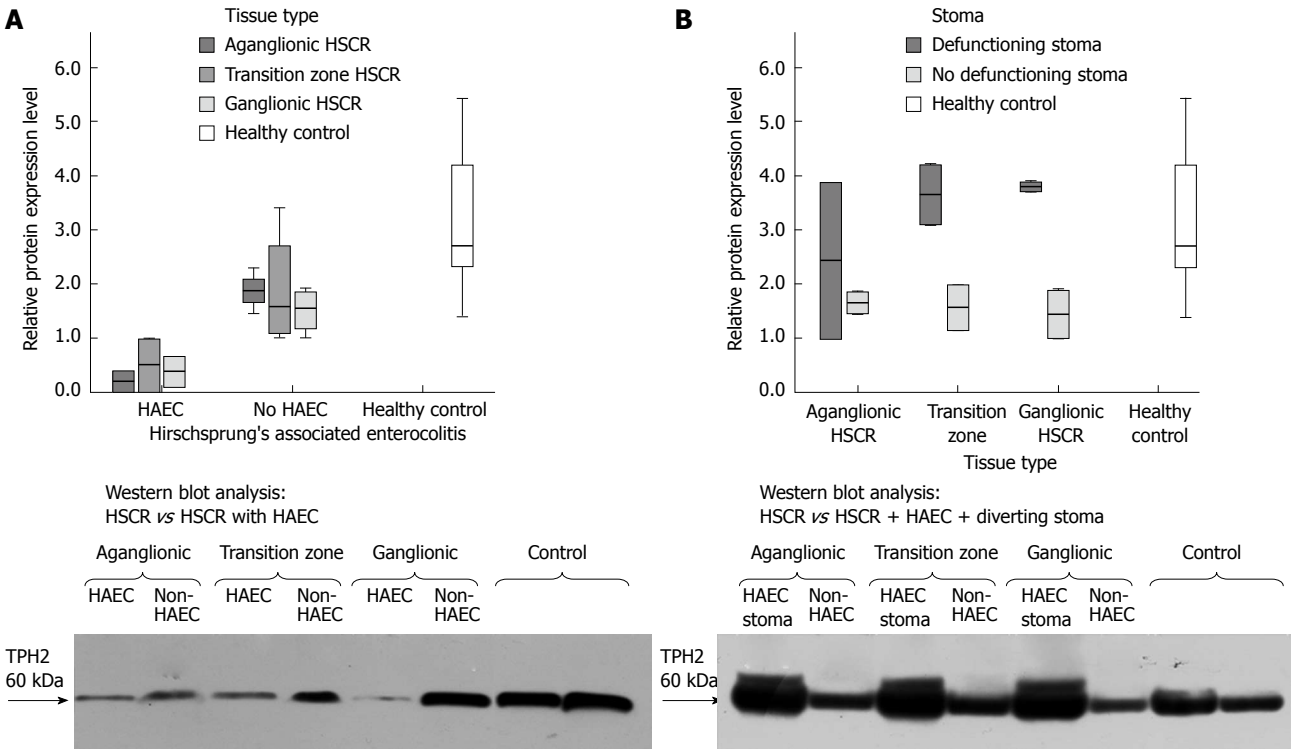

Figure 5 Western blot analysis. Western blot analysis is seen in (A) showing reduced expression of TPH2 in the colon of children with HSCR complicated by HAEC, who were managed non-operatively prior to pull-through surgery. The reduction in expression is seen across the aganglionic and ganglionic bowel of these patients. Image (B) shows the impact of diverting stoma formation on TPH2 expression in children with HSCR and pre-operative HAEC, with increased expression seen when compared to children without a history of HAEC and no stoma. In both (A) and (B), protein expression has been normalized against the loading control, GAPDH (36kDa). TPH2: Tryptophan hydroxylase-2; HSCR: Hirschsprung's disease; HAEC: Hirschsprung's-associated enterocolitis.

neurons and submucosal ICCs were richly supplied by serotonergic varicosities in murine colon and suggested a potential role for neuronal 5-HT in the regulation of slow wave electrical activity. Liu *et al*[16] demonstrated that 5-HT acts on the 5-HT$_3$ receptor on ICCs, wherein the receptor functions as a Ca^{2+}-influx mechanism to augment pacemaker activity in ICCs. The intimate spatial arrangement of serotonergic nerves with nNOS-positive nerves is also important as it is thought that, through the intercession of descending inhibitory serotonergic neuronal activity, the release of nitric oxide from nNOS-positive neurons suppresses excitatory cholinergic activity and limits the rate at which colonic migrating motor complexes (CMMC) are propagated in mice[14]. The equivalent peristaltic activity to the CMMC in humans is the high-amplitude propagating contraction.

A close spatial arrangement of myenteric ICC fibers with TPH2-positive ganglia was demonstrated in our study, with co-expression of TPH2 and nNOS in myenteric and submucosal nitrergic ganglia and submucosal ANO-1 positive ICCs. In addition, we have demonstrated co-expression of TPH2 in the relatively recently described PDGFRα^+ cells. These fibroblast-like cells share morphological similarities with ICCs but are c-kit negative[17]. It is thought that they play a role in transducing purinergic neurotransmission through the activity of apamin-sensitive small-conductance Ca^{2+}-activated K^+ (SK3) channels[18]. Our immunofluorescence findings suggest a possible functional role for serotonergic neurons in modulating PDGFRα^+ cell function. It is inferable from the current evidence in the literature, that reduced density of serotonergic nerves in aganglionic and transition zone bowel and, in some patients, the ganglionic bowel, in HSCR, would disturb the inhibitory mechanisms required to maintain normal colonic motility. This is of particular relevance in patients with HAEC.

Mucosal 5-HT, secreted by EC cells, has been demonstrated in animal models to be pro-inflammatory, probably due to its activation of 5-HT receptors on dendritic cells in the lamina propria[8]. In other inflammatory conditions of the bowel, such as ulcerative colitis, reduced levels of the SERT have been reported, leading to increased 5-HT availability[8]. Conversely, neuronal 5-HT has been shown to be neuroprotective. *In vitro* and *in vivo* studies have demonstrated that neuronal 5-HT acts *via* 5-HT$_{2B}$ and 5-HT$_{4A}$ receptors respectively to promote survival of ICCs and enteric neurons respectively[7,8].

It has long been recognized that significant enteric neural damage occurs in severe inflammatory conditions of the colon, particularly in the setting of necrotizing enterocolitis[19-21]. Our finding of reduced expression of TPH2 in the aganglionic, transition zone and ganglionic bowel in HSCR complicated by

HAEC, compared with patients unaffected by HAEC and controls, is probably reflective of enterocolitis-mediated neuronal damage. The vicious circle of enterocolitis and loss of neuroprotective serotonergic neurons may thus occur. We have previously reported on the outcomes of children with HSCR complicated by HAEC. It was found that approximately one third of patients in the series experienced enterocolitis both pre- and post-operatively[2]. Disturbances in colonic function were also more common in these patients at long-term follow-up[2]. That our findings represent effect rather than cause is supported by the finding that levels of TPH2 greatly recover in the defunctioned colon of children with HSCR complicated by HAEC who were treated with diverting colostomy due to failure of medical management.

One striking characteristic of the population from whom pull-through specimens were collected for this study is the high proportion of children with trisomy 21 at 41.2%. The incidence of trisomy 21 in Ireland, at 1 in 546 live births, is the highest in Europe[22]. Children with trisomy 21 are recognized to be at a considerably higher risk of developing HAEC (approximately 51%) compared to those without trisomy 21[23]. In our study 2 of the 5 patients who developed pre-operative HAEC had trisomy 21, both of whom were treated with a diverting stoma, indicating the severe nature of their enterocolitis.

In conclusion, we have demonstrated that serotonergic neurons co-express TPH2 with ANO1-immuno-positive ICCs in the submucosa, even in aganglionic bowel. We have also shown, for the first time, co-expression of TPH2 in PDGFRα^+ cells, suggesting a possible role for serotonergic modulation of their function. TPH2 expression was reduced in the myenteric plexus and deep smooth muscle layers of aganglionic colon in children with HSCR unaffected by pre-operative HAEC. However, in children with HSCR complicated by HAEC, TPH2 expression was reduced in both aganglionic and ganglionic bowel - a finding that was reversed in the colon of children treated with diverting stoma formation due to HAEC refractory to non-operative strategies. HAEC-mediated serotonergic neuronal damage may contribute to ongoing problems with colonic function and recurrent enterocolitis despite properly performed corrective pull-through surgery.

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COMMENTS

Background

Hirschsprung's disease (HSCR) is the most common congenital gut motility disorder. It may be complicated by a severe pancolitis known as Hirschsprung's-associated enterocolitis (HAEC). Despite constituting only approximately 1%-5% of total intestinal serotonin (5-HT), neuronal 5-HT plays a key role in modulating gut motility and is thought to have an anti-inflammatory, neuroprotective role, in contrast to mucosal 5-HT which is pro-inflammatory.

Research frontiers

Dysregulation of mucosal 5-HT transport has already been implicated in ulcerative colitis and other inflammatory disorders of the human colon. While a previous study has described a reduction of enterochromaffin cells, which produce mucosal 5-HT, in the ganglionic colon of children with a history of HAEC, neuronal 5-HT expression has yet to be evaluated in HSCR. In mice, knockout of tryptophan hydroxylase 2 (TPH2), the key enzyme in the synthesis pathway of neuronal 5-HT, leads to slow gastrointestinal transit and severe intestinal inflammation. Conversely, knockout of TPH1, the key enzyme in the synthesis of mucosal 5-HT, has no effect on gastrointestinal motility.

Innovations and breakthroughs

It is known that patients with HSCR who experience pre-operative HAEC are at an increased risk of poor functional outcome despite optimal surgical treatment, with some patients continuing to experience severe constipation and recurrent enterocolitis even in the absence of a mechanical obstruction. The causes for this have yet to be fully elucidated. Current findings by the authors suggest that neuronal 5-HT is deficient in the ganglionic colon of children with a history of pre-operative HAEC. Given its anti-inflammatory, neuroprotective roles, our findings suggest a mechanism by which post-operative enterocolitis may occur.

Applications

5-HT and its receptors are some of the most important current pharmacological targets in the alteration of gut motility. While the authors have indirectly shown a reduction in neuronal 5-HT in the healthy ganglionic colon in HSCR after HAEC, it is unclear if this finding would persist at follow-up after a pull-through operation, as expression of TPH2 appeared to have recovered in patients who underwent levelling or defunctioning colostomy. These findings lay the foundation for future work examining whether the abnormalities detected in this study persist at follow-up, as well an empiric evaluation of the functional outcomes of patients in this study.

Terminology

Tryptophan hydroxylase 2 (TPH2) is an enzyme which catalyzes the conversion of L-tryptophan to 5-hydroxytryptophan, which is the critical rate-limiting step of neuronal 5-HT synthesis. It is an isozyme of TPH1, which catalyzes the same reaction in the synthesis of mucosal 5-HT in enterochromaffin cells.

Peer-review

This is a clearly presented study, addressing an important aspect of the role of neuronal 5-HT in Hirschprung's disease.

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ORIGINAL ARTICLE

Basic Study

Steatotic livers are susceptible to normothermic ischemia-reperfusion injury from mitochondrial Complex-Ⅰ dysfunction

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Abstract

AIM: To assess the effects of ischemic preconditioning (IPC, 10-min ischemia/10-min reperfusion) on steatotic liver mitochondrial function after normothermic ischemia-reperfusion injury (IRI).

METHODS: Sixty male Sprague-Dawley rats were fed

8-wk with either control chow or high-fat/high-sucrose diet inducing > 60% mixed steatosis. Three groups (n = 10/group) for each dietary state were tested: (1) the IRI group underwent 60 min partial hepatic ischemia and 4 h reperfusion; (2) the IPC group underwent IPC prior to same standard IRI; and (3) sham underwent the same surgery without IRI or IPC. Hepatic mitochondrial function was analyzed by oxygraphs. Mitochondrial Complex-Ⅰ, Complex-Ⅱ enzyme activity, serum alanine aminotransferase (ALT), and histological injury were measured.

RESULTS: Steatotic-IRI livers had a greater increase in ALT (2476 ± 166 *vs* 1457 ± 103 IU/L, P < 0.01) and histological injury following IRI compared to the lean liver group. Steatotic-IRI demonstrated lower Complex-Ⅰ activity at baseline [78.4 ± 2.5 *vs* 116.4 ± 6.0 nmol/(min·mg protein), P < 0.001] and following IRI [28.0 ± 6.2 *vs* 104.3 ± 12.6 nmol/(min·mg protein), P < 0.001]. Steatotic-IRI also demonstrated impaired Complex-Ⅰ function post-IRI compared to the lean liver IRI group. Complex-Ⅱ activity was unaffected by hepatic steatosis or IRI. Lean liver mitochondrial function was unchanged following IRI. IPC normalized ALT and histological injury in steatotic livers but had no effect on overall steatotic liver mitochondrial function or individual mitochondrial complex enzyme activities.

CONCLUSION: Warm IRI impairs steatotic liver Complex-Ⅰ activity and function. The protective effects of IPC in steatotic livers may not be mediated through mitochondria.

Key words: Mitochondrial respiration; Fatty liver; Liver ischemia; Oxidative phosphorylation; Liver injury; Hepatic steatosis; Ischemic preconditioning

Core tip: We report a detailed mitochondrial function analysis of dietary-induced hepatic steatosis, which was not choline-deficient, during warm ischemia and after ischemia-reperfusion injury. We evaluated mitochondrial complex Ⅰ and Ⅱ activities as well as the impact of ischemic preconditioning on mitochondrial function. This study demonstrates that steatotic livers have decreased Complex-Ⅰ activity at baseline and that Complex-Ⅰ function is further impaired after warm ischemia-reperfusion injury. Ischemic preconditioning was unable to attenuate the harmful effect of ischemia-reperfusion on mitochondrial function.

Chu MJJ, Premkumar R, Hickey AJR, Jiang Y, Delahunt B, Phillips ARJ, Bartlett ASJR. Steatotic livers are susceptible to normothermic ischemia-reperfusion injury from mitochondrial Complex-Ⅰ dysfunction. *World J Gastroenterol* 2016; 22(19): 4673-4684 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i19/4673.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i19.4673

INTRODUCTION

Hepatic steatosis is the most common liver disease found in clinical liver biopsies[1], and autopsy-based studies estimate that the prevalence of hepatic steatosis is 15%-30% in the Western world[1]. Consequently, the number of patients with hepatic steatosis encountered during liver surgery is increasing. Hepatic steatosis has been associated with a 2-3 fold increase in post-operative complication rates following liver resection[2,3]. It has been proposed that steatotic livers are more susceptible to ischemia-reperfusion injury (IRI), which impairs liver regeneration and is a major cause of liver damage, leading to worse outcomes[3].

The exact mechanism for the increased susceptibility of steatotic livers to IRI is not fully understood. Steatotic livers have been shown to have decreased recovery of adenosine triphosphate (ATP) concentrations following IRI[4]. One of the proposed underlying mechanisms behind the decreased ATP recovery and increased steatotic liver susceptibility to IRI is mitochondrial dysfunction[5]. Mitochondria are responsible for producing the bulk of cellular ATP and are, therefore, fundamental for cellular viability[6]. Impaired mitochondrial function (MF) disrupts normal cellular bioenergetics, which leads to cell death[7].

To attenuate the deleterious effect of IRI, ischemic preconditioning (IPC) of the liver has been used[8]. IPC involves a brief period of ischemia followed by reperfusion (generally, 10 min ischemia and 10 min reperfusion) prior to a period of sustained ischemic insult[9,10]. IPC has been reported to improve post-IRI liver injury in experimental[9] and clinical[10] steatotic livers. IPC has also been reported to improve ATP levels in steatotic livers[11]. While the mechanism by which IPC improves hepatic outcome following IRI is unknown, it has been postulated that IPC modulates and somehow preserves MF[9]. The aim of this study was to evaluate the impact of IPC on the MF of rat livers with steatosis after being subjected to warm (normothermic) IRI. Mitochondrial bioenergetics and liver injury markers were evaluated.

MATERIALS AND METHODS

Animals

All reagents were purchased from Sigma-Aldrich (St. Louis, MO, United States) unless otherwise specified. All experiments were performed in 11-wk old male Sprague-Dawley rats. The animal protocol was designed to minimize discomfort to the animals. The animals were enrolled when 3-wk old and removed from the mother at this age. They were then randomized to receive standard chow (lean animals; Teklad TB 2018; Harlan, Madison, WI, United States; 18% kcal fat, 58% kcal carbohydrate) or a high-fat/high-sucrose diet (steatotic animals; Rodent Diet D03021303; Research Diets, Inc., New Brunswick, NJ, United States; 45 kcal% fat, 25% kcal sucrose; Table

Table 1 Content of high-fat/high-sucrose diet (D03021303; Research Diets, Inc., NJ, United States)

	Gram%	Kcal%
Protein	23.7	20
Carbohydrate	41.4	35
Fat	23.6	45
Total		100
Kcal/g	4.73	
Ingredient		
Casein, 80 Mesh	200	800
L-Cystine	3	12
Corn Starch	50	200
Maltodextrin 10	45.6	182
Sucrose	250	1000
Cellulose, BW200	50	0
Soybean Oil	25	225
Lard	177.5	1598
Mineral Mix S10026	10	0
DiCalcium Phosphate	13	0
Calcium Carbonate	5.5	0
Potassium Citrate. 1 H2O	16.5	0
Vitamin Mix V10001	10	40
Choline Bitartrate	2	0
FD and C Blue Dye #1	0.05	0
Total	858.15	4057

1)[12]. The animals were kept under a 12-h light/dark cycle (50%-70% humidity, 22 ± 2 ℃) with *ad libitum* access to food and water. Bodyweight and blood glucose were measured weekly. Rats were fasted for 6 h prior to surgery to mimic pre-operative fasting. All surgical procedures were started between 12:00-1:00 pm. All experiments were approved by the University of Auckland Animal Ethics Committee (R965).

Experimental design and surgical procedures

Sixty animals were randomized into one of six groups (n = 10 each): (1) Lean + Sham (Lean-Sham); (2) Lean + IRI (Lean-IRI); (3) Lean + IRI + IPC (Lean-IPC); (4) Steatotic + Sham (Steatotic-Sham); (5) Steatotic+IRI (Steatotic-IRI); and (6) Steatotic + IRI + IPC (Steatotic-IPC).

A model of partial (70%) hepatic ischemia was used that prevented mesenteric venous congestion by permitting portal decompression through the right and caudate lobes[9]. Rats were anesthetized with isofluorane inhalation. Following tracheostomy, anesthesia was maintained (1%-2% isofluorane) through a pressure-controlled ventilator (Kent Scientific Corporation, Torrington, CT, United States). Core body temperature was maintained (37-38 ℃) by a thermostatically-controlled warming plate. Fluid administration *via* the right femoral vein and mean arterial pressure monitoring *via* the right carotid artery were undertaken with a radio-opaque 22G catheter and 2F solid-state pressure transducer (SPR-320 pressure catheter; Millar Instruments Inc., Houston, TX, United States), respectively.

Following a transverse laparotomy, the hepatic artery and portal vein to the left and median lobes were occluded for 60 min with a microvascular clip. Removal of the clip initiated the 240 min of reperfusion. Rats receiving IPC received 10 min of ischemia and 10 min of reperfusion prior to 60 min of ischemia. In the sham group, the rats were anesthetized, and a laparotomy was performed for 5.5 h without induction of ischemia.

The placement of a tracheostomy (reflecting endotracheal intubation) and prolonged anesthesia mimic clinical liver resection whereby patients are anesthetized continuously. We did not perform short intervals of anesthesia and repeated mini-laparotomies for our protocol, as it does not reflect clinical practice.

Tissue collections

Liver samples were obtained by "cheese-wire" ligating the liver with 4-0 silk tie. This technique caused minimal bleeding from the cut surface of the liver and allowed repeated sampling from each rat. Liver samples were obtained at various time-points: "A": baseline (immediately following laparotomy), "B": 10 min (after 10 min ischemia), "C": 20 min (end of IPC), "D": 80 min (after 60 min ischemia), and "E": 320 min (end of 240 min reperfusion phase). Liver samples were obtained for histology, MF, and enzymatic analysis. The amount of liver tissue removed did not exceed 20% of total hepatic mass. At the end of the procedure, serum (5 mL) was collected from the inferior vena cava.

Histology

Histology was performed on time-points A and E to assess severity of the hepatic steatosis and IRI, respectively. Formalin-fixed and paraffin-embedded liver samples were stained with hematoxylin and eosin (HE). A consultant specialist histopathologist (BD) blinded to the group assessed the severity of steatosis with a published clinical grading system[13]. Severity of the IRI was assessed using a 4-point grading system previously described[14].

Assessment of hepatocyte injury

The severity of hepatic injury was assessed by serum levels of alanine aminotransferase (ALT) and was analyzed using a Roche Cobas 8000 modular analyzer (c702 module, Basel, Switzerland).

Homogenized tissue preparation for mitochondrial function analysis

Liver samples were immediately placed in ice-cold (about 4 ℃) mitochondrial respiration media (Table 2). The samples were then removed from the media, blotted, weighed (20-30 mg), and placed in a 2-mL flat-bottom scintillation vial with 500 μL of mitochondrial respiration media. The sample was homogenized for 5 s with an Omni TH homogenizer (Omni International, Kennesaw, GA, United States) before analysis. Homogenates were utilized as they

Table 2 Mitochondrial respiration media

Chemical	Final concentration (mmol/L)
EGTA	0.5
$MgCl_2$	3
K-Lactobionate	60
Taurine	20
KH_2PO_4	10
Sucrose	110
Bovine serum albumin	1 mg/mL
HEPES	20 (pH 7.0 at 37 ℃)

are more physiological and decrease the risk of any organelle selection bias inherent to the process of mitochondrial isolation, allowing for the preferential selection of more healthy organelles during isolation[15]. Our protocol also permits shorter processing time and rapid measurement of the function of the entire mitochondrial population within the tissue to provide an immediate measure of mass specific flux.

Mitochondrial respiration assays

Mitochondrial respiration of liver homogenate was measured at each time-point. Respiration was measured in 2-mL chambers using an OROBOROS Oxygraph 2K (Anton Paar, Graz, Austria) at 37 ℃ in mitochondrial respiration media, with a calculated saturated oxygen concentration of 190 nmol O_2 per milliliter at 100 kPa barometric pressure, and oxygen flux calculated using the DatLab 5 analysis software. Liver homogenate (50 μL) was added to each chamber, and the remainder was stored at -80 ℃ for later analysis. To account for potential variations in mitochondrial mass, citrate synthase (CS)-normalized[16] oxygen flux (pmol $O_2.s^{-1}.U\ CS^{-1}$) was calculated.

A multiple substrate-inhibitor titration protocol was used to explore relative contributions of complex Ⅰ (CI), complex Ⅱ (CⅡ), and combined CI+CⅡ in the electron transport system (ETS). Respiration states were defined according to Gnaiger[17], where leak respiration and oxidative phosphorylation (OXPHOS) were the flux measured before and after addition of adenosine diphosphate (ADP), respectively. The assay protocol steps are described in Table 3. The integrity of tissue preparations and comparison of coupling efficiencies were made from the respiratory control ratio (RCR).

Citrate synthase and total protein measurement

Liver homogenate, as used in mitochondrial respiration assay, was analysed for CS activity and total protein content. CS activity was measured as a surrogate for mitochondrial mass[16]. Frozen (-80 ℃) liver homogenate was thawed, and CS activities were determined following the method of Srere and modified to microtitre-plate[18]. Protein content was determined by the Biuret test with bovine serum albumin as standard.

Complex Ⅰ and Complex Ⅱ activities

Liver homogenate from time-points A, D, and E were used to measure individual CI and CⅡ enzyme activities using the NADH-oxidation and dichlorophenolindophenol-oxidation method, respectively[19]. Frozen liver homogenate was thawed and centrifuged for 10 min at 600 *g* (4 ℃), and the supernatant used for analysis. Assays were conducted using a 96-well format, and results were normalized to total protein content, as determined by the Biuret method. In this report, "CI function" refers to assessments undertaken in the oxygraph, and "CI activity" refers to the isolated activity studies just described.

Statistical and data analysis

All study data were recorded in an EXCEL database. Statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA, United States) and SAS version 9.2 (SAS institute, Cary, NC, United States). Statistical tests were set at a 5% significance level (two-sided). Student's *t*-tests were conducted on body weight and random blood glucose level to compare obese and lean rats. Difference in outcome measure between the groups of interest was tested using the analysis of covariance regression, adjusting for baseline value, bodyweight, and blood glucose measured before the procedures as appropriate. Repeated measures mixed model was used to evaluate the treatment differences at different time-points, controlling for the correlation of data collected on the same animal. The results are presented as mean ± SE of mean, with associated *P*-value.

RESULTS

Rats fed high-fat/high-sucrose diet were obese

Sprague-Dawley rats fed high-fat/high-sucrose diet for 8 weeks showed increased body weight (507 ± 10 *vs* 437 ± 6 g, $P < 0.0001$; Figure 1A) and increased random blood glucose level (6.1 ± 0.1 *vs* 4.6 ± 0.1 mmol/L, $P < 0.0001$; Figure 1B) relative to age-matched lean rats.

Obese rat livers had severe mixed steatosis

Obese rat livers had gross macroscopic fat accumulation. All lean rat livers had normal baseline underlying tissue architecture with only some mild (8% ± 1%) microvesicular steatosis when evaluated by H&E staining (Figure 1C). Obese rat livers had severe (65% ± 3%) baseline mixed steatosis with prominent macrovesicular steatosis features evident (Figure 1D). There were no signs of fibrosis or inflammation in any of the groups consistent with hepatic steatosis.

Steatotic livers had increased liver injury following ischemia-reperfusion

Effect of steatosis, IRI, and IPC on liver injury biomarkers,

Table 3 Mitochondrial respiration assay protocol

Reagents added	Final concentration in oxygraph chamber (mmol/L)	Action of reagent	Measurement output
Step 1			
Glutamate	10	CI substrates	CI leak respiration
Malate	5		(CI_{Leak})
Pyruvate	10		
Step 2			
ADP	1.25	Substrate for ATP generation	CI oxidative phosphorylation
Step 3			
Succinate	10	CⅡ substrate	CI+CⅡ oxidative phosphorylation[1]
Step 4			
Rotenone	0.001	CI inhibitor	Isolate flux to CⅡ [CⅡ(rot)]
Step 5			
Oligomycin	0.0025	ATP-Synthase inhibitor	CI+CⅡ leak respiration ($CI,_{ⅡLeak}$)
Step 6			
FCCP	0.0015	Mitochondrial uncoupler	ETS capacity
Step 7			
Antimycin A	0.0050	CⅢ inhibitor	Residual oxidase consumption

[1]The individual contribution of CⅡ to oxidative phosphorylation (CⅡ-OXPHOS) can also be derived (CI+Ⅱ-OXPHOS minus CI-OXPHOS). ADP: Adenosine diphosphate; ATP: Adenosine triphosphate; CI: Complex Ⅰ; CI-OXPHOS: Complex Ⅰ oxidative phosphorylation; CI+Ⅱ-OXPHOS: Complex Ⅰ + Complex Ⅱ oxidative phosphorylation; $CI,_{ⅡLeak}$: Complex Ⅰ + Complex Ⅱ leak respiration; CI_{Leak}: Complex Ⅰ leak respiration; CⅡ: Complex Ⅱ; CⅢ: Complex Ⅲ; ETS: Electron transfer system; FCCP: Carbonylcyanide *p*-trifluoromethoxy-phenylhydrazone; Rot: Rotenone.

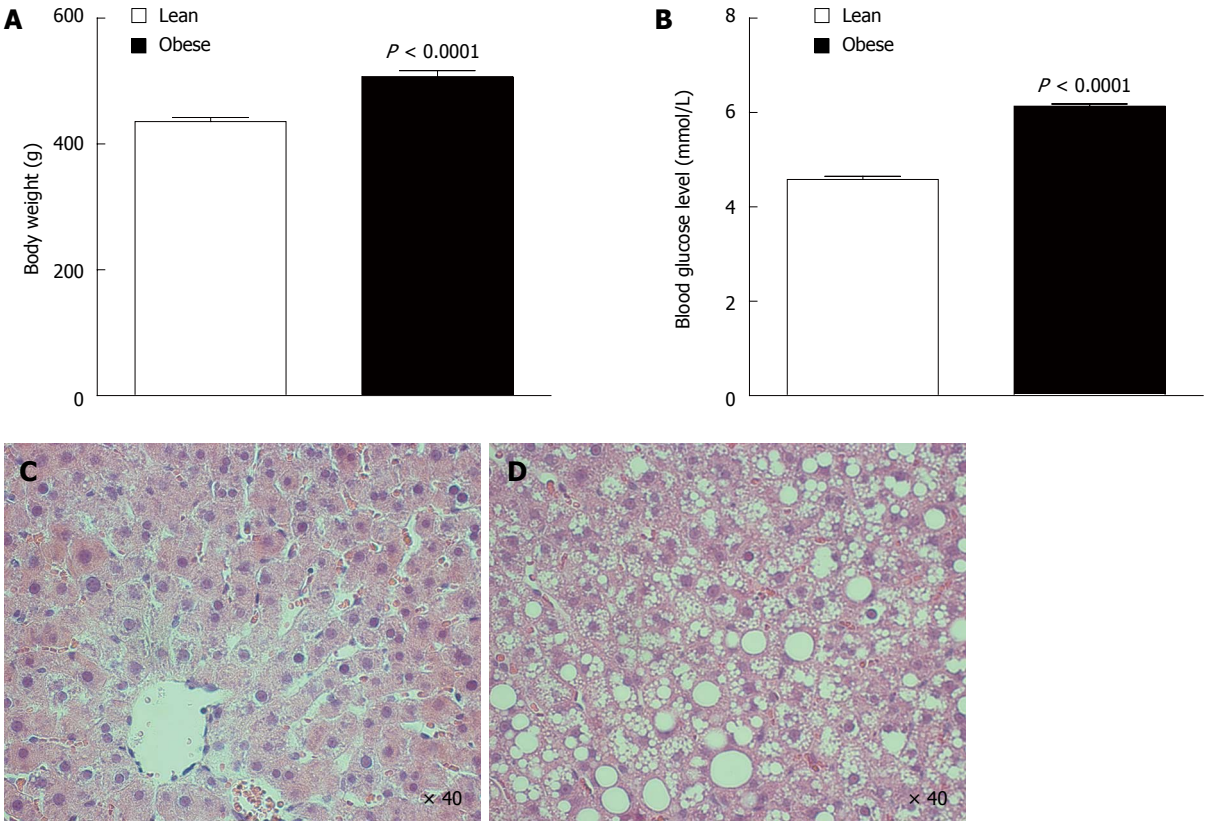

Figure 1 Bodyweight, random blood glucose, and baseline histology of high-fat/high-sucrose-fed (obese) and lean Sprague-Dawley rats. Obese Sprague-Dawley rats were significantly heavier (A) with higher random blood glucose (B) than age-matched lean rats. Baseline liver tissue sections were stained for hemotoxylin and eosin (x 40 magnification). Representative slides are displayed, and obese rats (D) showed severe mixed hepatic steatosis while lean rat livers (C) showed mild microvesicular steatosis. Data are shown as mean ± SE (n = 30 rat/group). Statistical analyses were performed using Students t-tests for body weight and random blood glucose.

tissue injury scores, and histology are shown in Figure 2 and 3, respectively. Both Lean-Sham and Steatotic-Sham livers did not have any biochemical (Figure 2A) or histological evidence of injury induced by the sham surgery (Figures 2B, 3A and 3B). Conversely, IRI was associated with increased serum ALT (Figure 2A) and worse liver histology injury scores in both Steatotic-IRI and Lean-IRI rats compared to Steatotic-Sham and

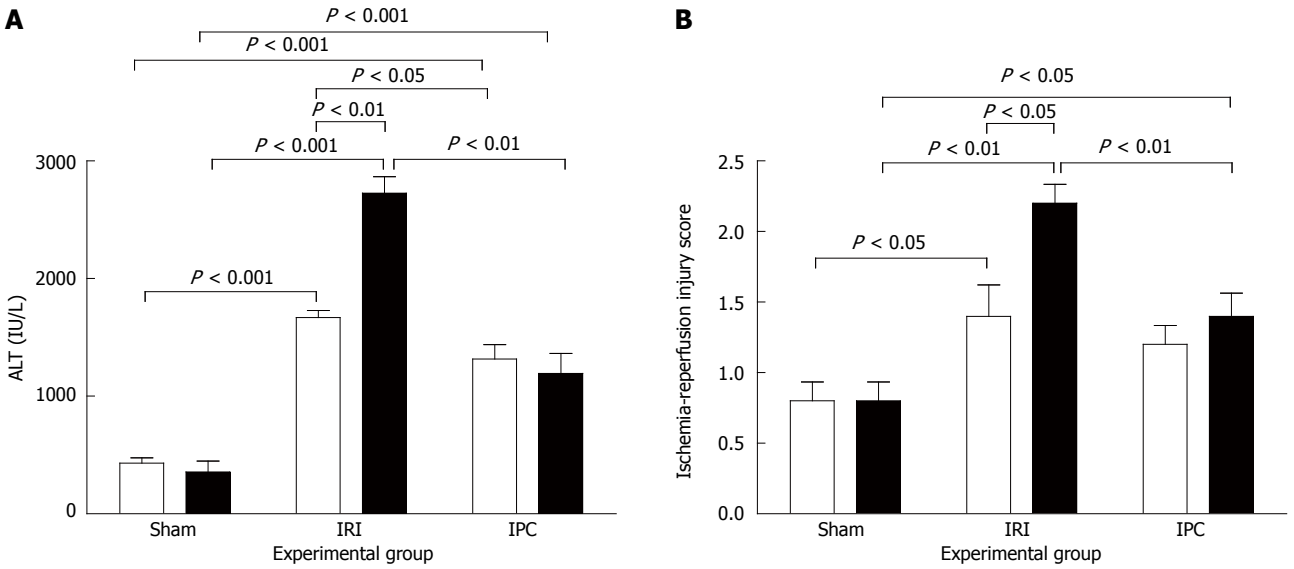

Figure 2 Serum alanine aminotransferase levels and histology injury score following ischemia-reperfusion. Serum alanine aminotransferase (ALT) (A) and histology injury score (B) following reperfusion were significantly higher in rats subjected to IRI compared to sham rats. Both injury markers were significantly higher in obese rats compared to lean rats. Compared to corresponding IRI groups, IPC decreased ALT levels in both lean and obese rats and decreased injury score in obese rats. Data are shown as mean ± SEM (n = 10 rat/group; lean rats, open bar; obese rats, closed bar). IRI: Ischemia-reperfusion injury; IPC: Ischemic preconditioning.

Lean-Sham rats, respectively (Figure 2B). These same injury markers were also found to be significantly higher in Steatotic-IRI rats compared to Lean-IRI rats (Figure 2). IPC led to improvement in the serum ALT in Steatotic-IPC and Lean-IPC rats compared to Steatotic-IRI and Lean-IRI rats, respectively (Figure 2A). IPC also decreased injury score in Steatotic-IPC rats compared to Steatotic-IRI rats (Figure 2B). These results indicate that IPC was able to attenuate liver injury in steatotic livers.

Baseline mitochondrial function in steatotic livers were similar to lean livers

The baseline mitochondrial functions were similar between steatotic and lean rat livers. Baseline (T = 0) samples from steatotic rat livers (n = 30) had similar MF to lean rat livers (n = 30) (Figure 4A-F). These results indicate that steatotic liver mitochondria were initially functioning adequately *in vivo*.

Sham-operated rat liver mitochondrial function remained stable

There were no changes in MF in both Lean-Sham and Steatotic-Sham livers with a stable CI-OXPHOS, CⅡ-OXPHOS, and RCR throughout all time-points (Figure 4A-F). These data indicate that the act of repeated liver sampling from each rat did not in itself significantly influence the underlying MF.

Prolonged ischemia led to impaired mitochondrial function

At the end of 60 min of ischemia, both lean and steatotic livers demonstrated impaired MF with significantly lower CI-OXPHOS (about 30%-40%), CⅡ-OXPHOS (45%-60%), and RCR (about 60%-80%) compared to pre-ischemic levels or corresponding Sham livers (Figure 4A-F). There was no observable difference in MF between Lean-IRI and Steatotic-IRI livers at the end of ischemia. These findings indicate impaired MF occurs to the same extent in all groups immediately following 60 min of ischemic insult.

Reperfusion injury led to decreased Complex Ⅰ mediated respiration in steatotic livers

After 60 min of ischemia and 240 min of reperfusion, MF in Lean-IRI returned to pre-ischemic levels and was comparable to Lean-Sham livers (Figure 4A, C, E). In Steatotic-IRI livers, CI-OXPHOS flux and RCR were significantly lower compared to baseline levels or Steatotic-Sham livers (Figure 4B, F) whereas CⅡ-OXPHOS flux rates returned to pre-ischemic levels and was comparable to Steatotic-Sham livers (Figure 4D). CI-OXPHOS flux rates and RCR in Steatotic-IRI livers were observed to be 57% and 54% relative to Lean-IRI livers post-reperfusion (P < 0.01). These results indicate that, unlike lean livers, steatotic liver CI function was impaired by IRI, leading to decreased RCR.

Ischemic preconditioning had no significant effect on hepatic MF

MF in both types of livers subjected to IPC was similar to livers subjected to IRI only (Figure 4A-F). Lean-IPC and Steatotic-IPC livers demonstrated a similar pattern of CI-OXPHOS, CⅡ-OXPHOS, and RCR at each of the sampling time-points as Lean-IRI and Steatotic-IRI livers, respectively (Figure 4A-F). Post-reperfusion, CI-OXPHOS, and RCR in Steatotic-IPC livers remained impaired, while CⅡ-OXPHOS was comparable to pre-ischemic levels. These results indicate that despite the improvement in injury markers (ALT and histology)

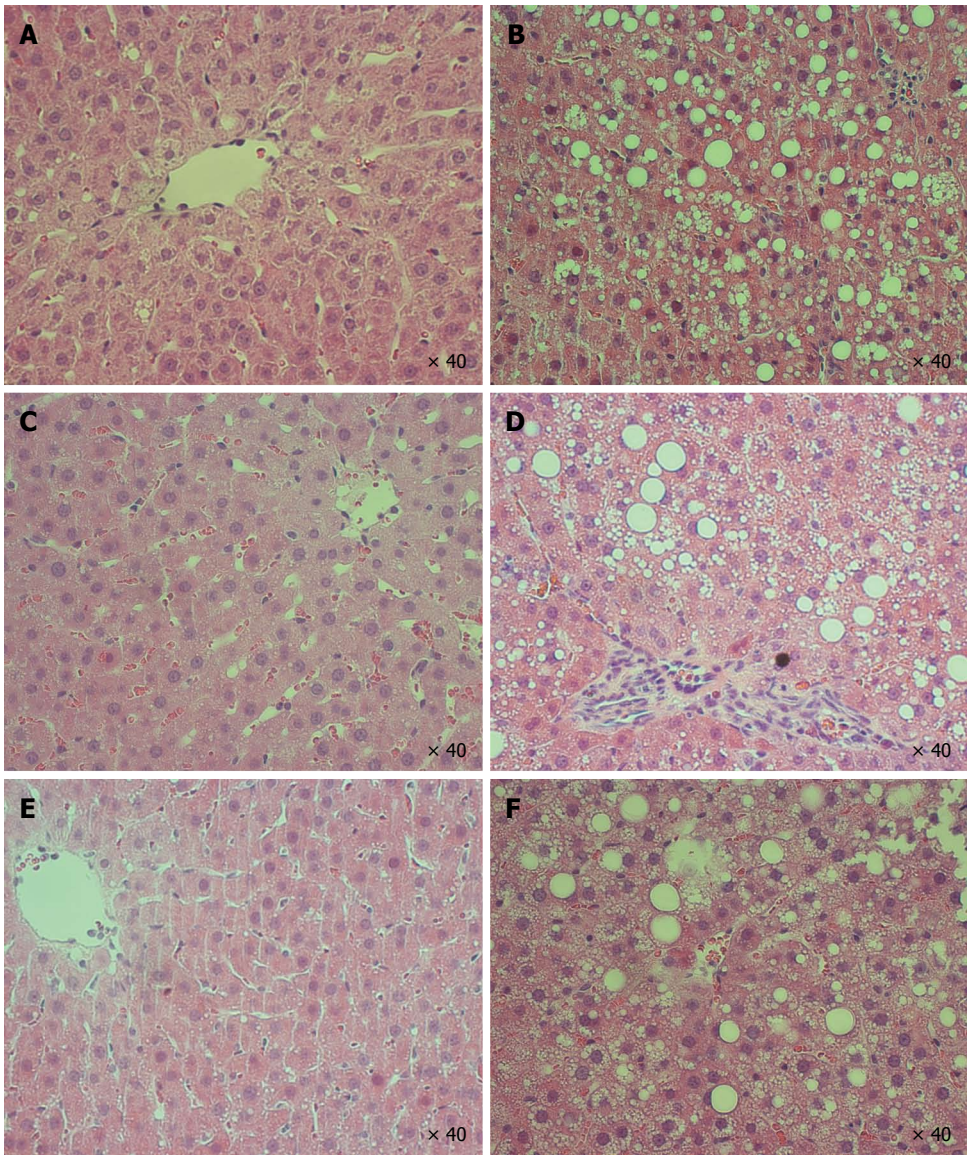

Figure 3 Liver histology following ischemia-reperfusion. No evidence of injury was observed in Lean-Sham (A) and Steatotic-Sham (B) livers. Lean-IRI livers (C) had mild injury while Steatotic-IRI livers (D) had moderate-severe injury. Both Lean-IPC (E) and Steatotic-IPC livers (F) were observed to have mild injury following reperfusion. Representative slides are shown (n = 10 rat/group). IRI: Ischemia-reperfusion injury; IPC: Ischemic preconditioning.

in IPC livers (Figures 2 and 3), IPC did not influence underlying MF over this timeframe.

Citrate synthase activity was unaffected by ischemia-reperfusion injury

Citrate synthase (CS) activity in lean and steatotic livers was stable throughout the experiment and was not affected by IRI or IPC (Figure 5A and B). There was also no difference in CS activity between lean and steatotic livers at all time-points measured.

Decreased Complex Ⅰ but not Complex Ⅱ enzymatic activity following reperfusion in steatotic livers

Baseline CI activity was significantly lower in steatotic livers compared to lean livers [78.4 ± 2.5 *vs* 116.4 ± 6.0 nmol/(min·mg protein), $P < 0.001$], while baseline CⅡ activity was similar between the two groups [104.9 ± 3.3 *vs* 116.8 ± 6.1 nmol/(min·mg protein), $P = 0.08$; Figure 5C and D]. Following 60 min of ischemia, both types of liver demonstrated significantly lower CI activity (Figure 5C and D) compared to pre-ischemic or sham livers. Steatotic liver CI activity was also observed to be lower post-ischemia compared to lean livers. Following reperfusion, CI activity returned to pre-ischemic levels in lean livers but remained significantly lower in steatotic livers by approximately 65% (Figure 5D). CⅡ activity (Figure 5E and F) was stable throughout the procedure, and there was no difference in CⅡ activity between steatotic and lean livers. IPC did not have any significant beneficial effect on CI and CⅡ activity in both types of livers. These activity results showed that IRI led to decreased CI activity in steatotic livers and also that IPC was not able to influence CI or CⅡ activities; all of which was

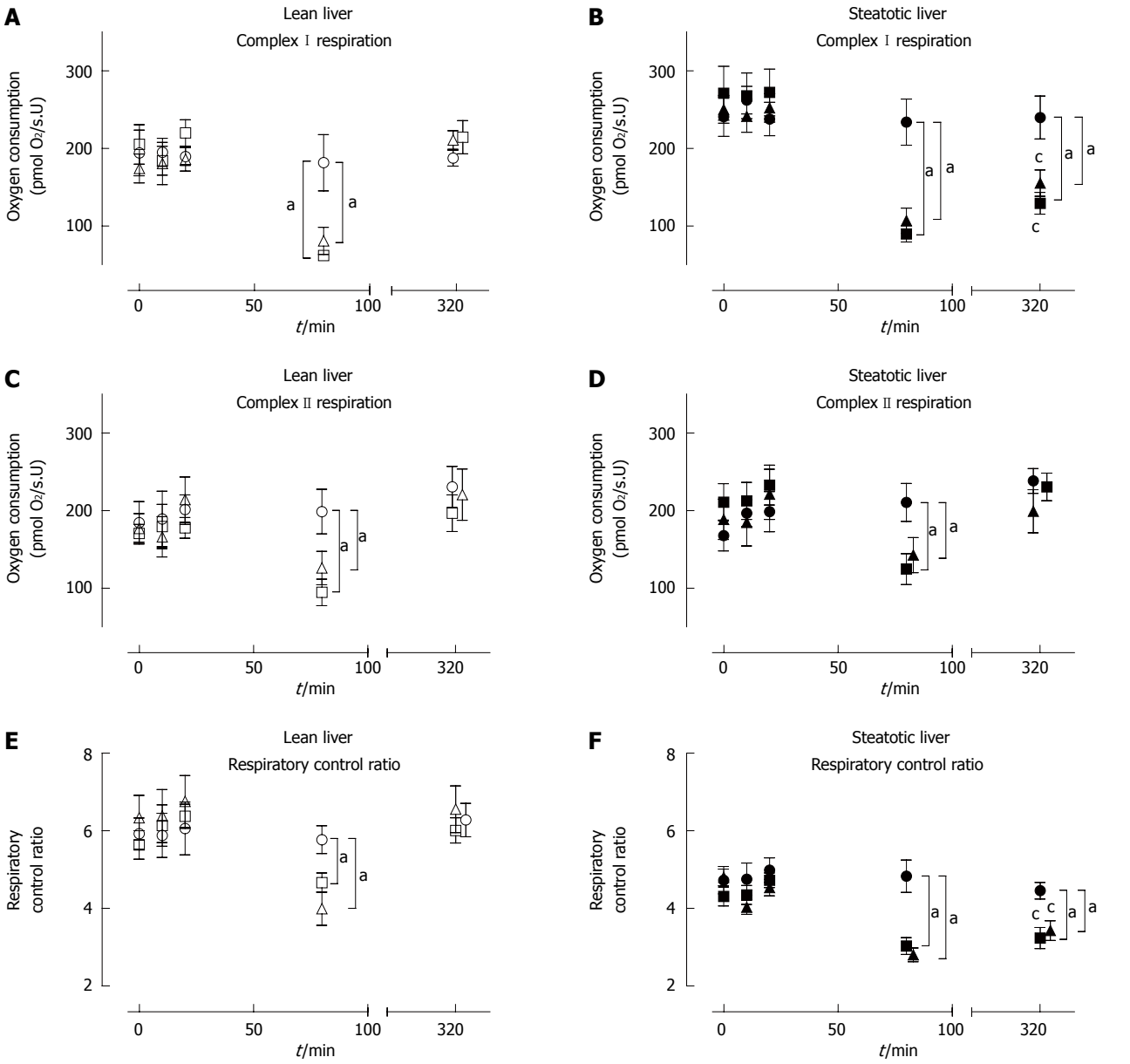

Figure 4 Mitochondrial function of lean and steatotic livers subjected to sham or ischemia-reperfusion injury with or without ischemic preconditioning. Baseline MF was similar between lean and steatotic livers in all outcome measures. Lean-Sham and Steatotic-Sham had stable CI-OXPHOS (A, B), C Ⅱ-OXPHOS (C, D), and RCR (E, F) throughout the procedure. CI-OXPHOS (A, B), C Ⅱ-OXPHOS (C, D), and RCR (E, F) were significantly lower following 60 min of ischemia in Lean-IRI, Lean-IPC, Steatotic- IRI, and Steatotic-IPC livers compared to the corresponding sham group. Following reperfusion, CI-OXPHOS (B) and RCR (F) were significantly lower in Steatotic-IRI and Steatotic-IPC livers compared to Steatotic-Sham or lean livers, while C Ⅱ-OXPHOS (D) returned to pre-ischemic levels comparable to Steatotic-Sham or lean livers (D). Data are shown as mean ± SE (n = 10 rat/group; Lean-Sham, open circle; Lean-IRI, open square; Lean-IPC, open triangle; Steatotic-Sham, closed circle; Steatotic-IRI, closed square; Steatotic-IPC, closed triangle). [a]$P < 0.05$ *vs* Lean-IRI; [c]$P < 0.05$ *vs* Lean-IPC (end of reperfusion). IRI: Ischemia-reperfusion injury; IPC: Ischemic preconditioning.

consistent with the earlier oxygraph functional analysis (above).

DISCUSSION

In this study, we used Sprague-Dawley rats with diet-induced hepatic steatosis. Compared to normal lean livers, steatotic livers demonstrated increased parenchymal injury following IRI, as indicated by their raised serum ALT and histology injury scores. Steatotic livers had lower baseline CI activity but similar baseline CS and C Ⅱ activity compared to lean livers. The steatotic livers were also observed to have decreased CI activity and function following IRI, which unlike lean livers, showed no recovery of either activity or function even after prolonged reperfusion times. This finding indicated that CI is a particularly vulnerable site of IRI-induced damage in steatotic livers. Our results demonstrated that IPC was effective in decreasing liver injury in both lean and steatotic livers according to ALT levels. However, this protective effect was not translated to measures of CI function or CI activity. In summary, these data demonstrate that steatotic livers developed significant underlying mitochondrial

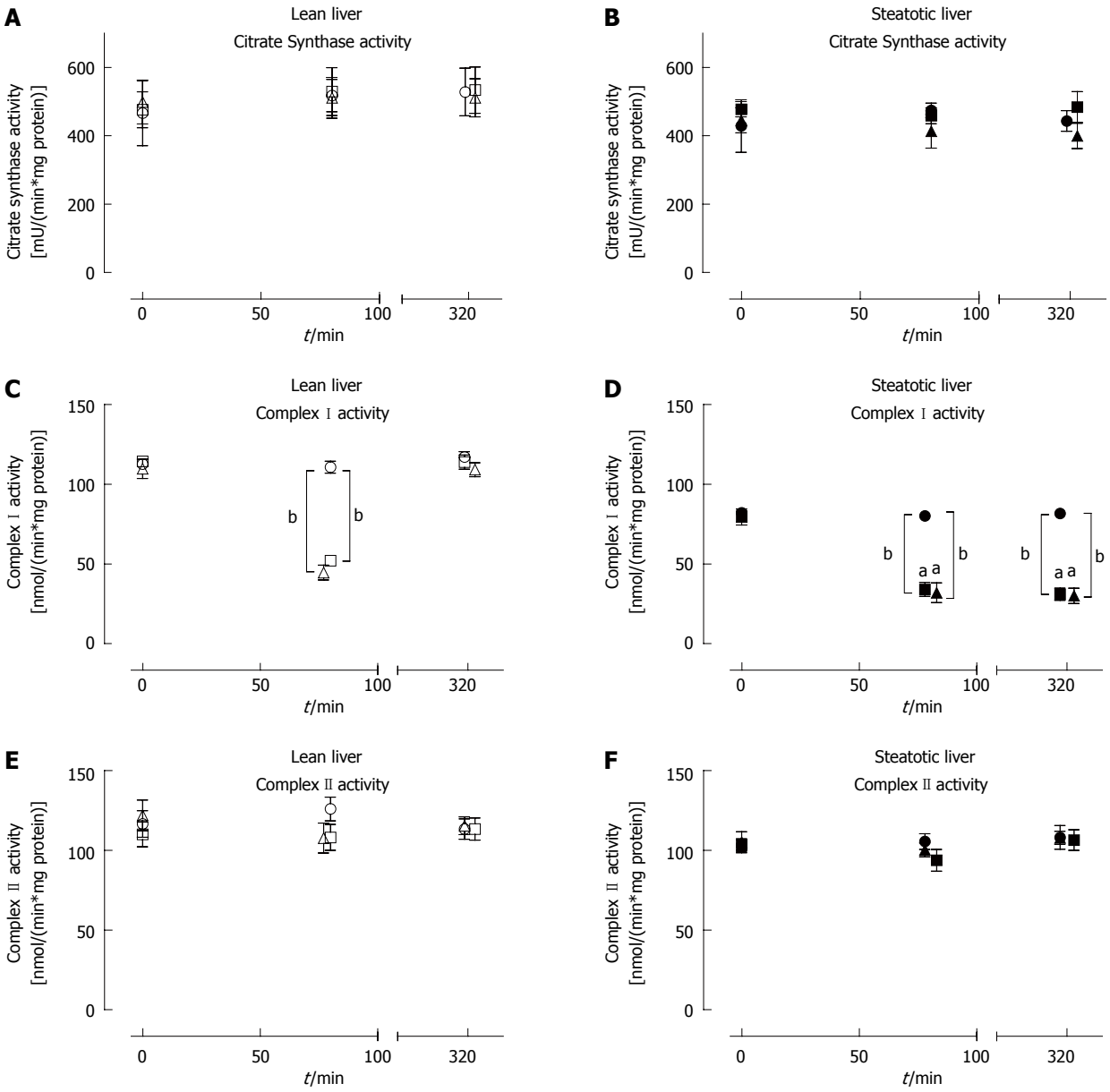

Figure 5 Citrate synthase, Complex Ⅰ and Complex Ⅱ activity at baseline, following ischemia and post-reperfusion in lean and steatotic livers. CS activity was similar between lean (A) and steatotic livers (B) throughout the procedure and was not affected by IRI or IPC. Baseline CI enzyme activity in steatotic livers were lower than lean livers (C, D). After ischemia, CI enzyme activity was significantly lower in lean and steatotic livers compared to sham livers. Additionally, CI enzyme activity (D) was lower in steatotic livers post-ischemia compared to lean livers. After reperfusion, CI activity remained lower in Steatotic-IRI and Steatotic-IPC livers (D) compared to Steatotic-Sham or lean livers. CⅡ activity (E, F) remained stable throughout the procedure and was similar between both types of livers. IPC did not have a significant effect on CI or CⅡ activity in both types of livers. Data are expressed as mean ± SE (n = 10 rat/group; Lean-Sham, open circle; Lean-IRI, open square; Lean-IPC, open triangle; Steatotic-Sham, closed circle; Steatotic-IRI, closed square; Steatotic-IPC, closed triangle). $^{a}P < 0.05$, $^{b}P < 0.001$ *vs* time- and group-matched lean livers. CS: Citrate synthase; CI: Complex Ⅰ; CⅡ: Complex Ⅱ; IRI: Ischemia-reperfusion injury; IPC: Ischemic preconditioning.

impairment that was worsened by IRI and not able to be recovered by IPC.

In this study we developed a novel methodology for the repeated procurement of liver samples from the same animal over time, which has not been published before. This technique not only decreased the number of animals used but also presents statistical advantages by enabling repeated measures analyses to identify study effects. The theoretical disadvantage of the progressive hepatectomy samples altering the status of the subsequent samples did no eventuate. In particular, when the Sham groups were examined, there were no significant changes found in serum ALT, histological scores, or MF using the progressive sampling approach.

MF and complex enzyme activities

Steatotic livers had impaired MF, which is thought to contribute to the increased steatotic liver susceptibility to IRI[5]. CS activity was similar across groups and time-points, indicating that MF differences in this study are due to alterations in mitochondria activity and not

a difference in mitochondrial mass.

A key dysfunction was found in CI, which is a large protein (about 1 MDa) comprising 45-47 subunits. It is embedded in the mitochondrial inner membrane to form an essential component of the mitochondrial ETS[20]. Impaired CI function has a substantial effect on ATP generation and contributes to a wide range of pathologies[21]. Our MF findings are consistent with a previous study that reported similar baseline CI function between steatotic and control livers and lower post-reperfusion CI function in isolated mitochondria from steatotic livers[5]. We have now extended this finding to show that the individual CI activity in tissue homogenates was also affected in steatotic livers post-IRI. Steatotic liver CI has previously been shown to be susceptible to oxidative damage from decreased mitochondrial antioxidants[5]. Further exacerbating this effect, CI can be a major site of reactive oxygen species production[22], and steatotic livers produce more reactive oxygen species *in vivo* than lean livers[5]. Oxygen reperfusion post-ischemia also leads to greater superoxide generation in steatotic livers relative to lean livers[23]. The lower CI activity observed in steatotic livers may be due to damage from IRI or may be a physiological response of steatotic livers to limit oxidative mitochondrial damage; which may be contributing to the decrease in tolerance of steatotic livers to IRI.

Complex Ⅱ is the only mitochondrial membrane-bound enzyme that is also involved in the citric acid cycle, as it oxidizes succinate and transfers electrons to co-enzyme Q[24]. CⅡ function has been reported to be similar at baseline and post-IRI in steatotic and lean rat livers[5], and our results corroborate this finding. CⅡ abnormalities are infrequently reported in the literature[19], and its function in steatotic livers are seldom reported. We observed that CⅡ activity post-ischemia and post-reperfusion was similar between steatotic and lean livers, which had not been previously described in this context. CⅡ activity and function appears to be unaffected by IRI, suggesting that CⅡ is more resistant to damage than CI. CⅡ may even contribute to superoxide production through an apparent reverse electron flow to CI instead[24], although this scenario is somewhat controversial, as it appears to defy thermodynamics and redox potentials[21]. Despite intact CⅡ function, CⅡ-driven flux is less effective in ATP synthesis (coupled to OXPHOS at Complex Ⅲ and Complex Ⅳ) compared to CI-driven flux (coupled to OXPHOS at CI, Complex Ⅲ and Complex Ⅳ), and this reduction in CCI-driven flux should impair reconstitution of ATP pools on reperfusion.

Effect of ischemic preconditioning

MF recovery following IRI is thought to be essential, as it generates the majority of cellular ATP[6]. Inadequate MF post-IRI would lead to decreased or delayed ATP generation during the critical period of reperfusion and could impair liver recovery. In this study, we hypothesized that IPC protects the steatotic liver from IRI-induced damage by protecting MF. We showed that IPC was partially protective against normothermic IRI (biochemical and histological indices) in both lean and steatotic livers, consistent with other studies[9,11]. In our study, liver transaminase levels were improved in preconditioned steatotic livers, which had not been previously investigated in diet-based models of obesity. These results were consistent with the limited clinical data on the effect of IPC on biochemical markers from steatotic livers subjected to normothermic IRI during liver resection[10,25].

Our results also indicated that despite some improvement in conventional liver injury markers, IPC did not improve MF or key enzyme activities over the duration of the study. This finding was in contrast to results from a previous study in choline-deficient rats that showed improved conventional liver injury markers and MF in preconditioned steatotic livers post-IRI[9]. Importantly, there were substantial differences in our study design to that of the only other previous experimental study. In that study, Rolo *et al*[9] performed MF analysis on isolated mitochondria at 25 ℃ and demonstrated that RCR was lower in both lean and steatotic livers post-IRI, and these effects were normalized by IPC in both groups of livers. However, mitochondrial respiration and particularly State 4 respiration are sensitive to assay temperature[26], and the results may not be truly representative of physiological MF at 37 ℃. Furthermore, the process of mitochondrial isolation results in the loss of fragile and/or damaged mitochondrial sub-populations. Here, we decided to use tissue homogenates as a means to lessen the potential for any mitochondrial selection bias[15]. Additionally, animals fed the choline-deficient diet used by Rolo *et al* showed weight loss, which is in contrast to the obesity often seen clinically in patients with hepatic steatosis[27]. In this study, the combination of a dietary model, tissue homogenates, and undertaking MF analysis at 37 ℃ represented the first advancement to a more physiological and clinically relevant MF analysis of the interaction of steatosis, IRI, and IPC. It is of note that most other previous studies on IPC in steatotic livers were performed in genetic models of hepatic steatosis[28]. However, the underlying mutations in these models are not prevalent in clinical hepatic steatosis pathophysiology, and the high-fat/high-carbohydrate used in our study more closely resembles the clinical setting[27].

The mechanism of IPC has been extensively reviewed elsewhere[29], but the direct impact of IPC on mitochondria is not as well characterized. In experimental lean rat livers subjected to IRI, ATP recovery was unaffected by IRI; but in steatotic livers, ATP recovery was found to be impaired post-IRI compared to lean livers[30]. In other studies, IPC was reported to preserve ATP recovery in lean livers post-IRI[29], while other studies have suggested that IPC was

also effective in improving ATP recovery in steatotic livers post-IRI[11]. For our study, we investigated MF and complex activities. We chose a 10′ + 10′ IPC protocol, as this was similar to the first clinical protocol described by Clavien *et al*[10]. In their study, IPC led to an improvement in biochemical and histological markers of injuries. This finding was then replicated in a further prospective trial[25], which demonstrated that IPC improved ATP levels in younger patient's post-reperfusion. In older patients, however, IPC decreased ATP levels post-reperfusion when compared to control livers. We found no improvement in MF with IPC, and although we were not able to measure ATP in this study, our findings suggest that the protective effect of IPC in this model was not likely to be mediated through increased mitochondrial ATP production. Therefore, there may be other mechanisms underlying the effect of IPC on hepatic ATP recovery. These mechanisms may include decreased cellular metabolism in preconditioned livers leading to conservation of ATP or reduced microcirculatory dysfunction. Alternatively, some of the IPC benefit may be due to increased production of nitric oxide and to opening of ATP-dependent potassium channels in preconditioned livers with a subsequent decrease in energy consumption[29].

The lack of full protection and liver function recovery from IPC observed by us and others in clinical and animal studies may reflect persistent underlying mitochondrial dysfunction, as demonstrated in our present study[3]. This observation supports future investigation of other IPC protocols or combinatorial use with mitochondrial-targeted therapies, as these may provide further clinical improvements. However, it could also potentially be influenced by our animal model, as our model differs from those published in the literature[9]. For completeness, liver weights should have been measured, but samples were obtained in "piece-meal" fashion and its was not possible.

As the prevalence of metabolic syndrome continues to rise in the population, hepatic steatosis has become the most common hepatic abnormality[1]. Therefore, it is important to identify new ways to improve outcomes from steatotic liver surgery. In this study, we investigated the impact of IPC on MF in a steatosis setting. We demonstrated that IRI was associated with increased liver injury in steatotic livers. Although the precise mechanisms underlying the increased susceptibility of steatotic liver to IRI remain unclear, we have shown for the first time, using a clinically relevant diet model and MF analysis at physiological temperatures, that there was an inherent decreased in CI activity in steatotic livers, which worsened following IRI. Our results also showed that the IPC protocol used in our study, while improving liver biomarkers and histology, did not influence MF directly. If we are to improve further the clinical benefit of IPC on livers with steatosis, then testing alternative IPC protocols or adjunct mitochondrial therapies will also be needed.

COMMENTS

Background

Steatotic livers are encountered with increasing frequency in liver surgery. It has been associated with poor outcome following warm ischemia-reperfusion injury (IRI). One possible proposed mechanism was mitochondrial dysfunction. However, the relationship between hepatic steatosis and mitochondrial dysfunction in warm IRI has not been clearly defined. Ischemic preconditioning has been touted as a possible therapeutic option for attenuating the harmful effect of IRI.

Research frontiers

Mechanisms of injury in steatotic livers are poorly understood, and further understanding will improve patient outcome.

Innovations and breakthroughs

This study is the first to investigate mitochondrial function, mitochondrial Complex Ⅰ and Complex Ⅱ activities; and the impact of ischemic preconditioning on mitochondrial function in warm IRI, in a dietary-induced model of hepatic steatosis.

Applications

Steatotic livers have decreased baseline Complex Ⅰ activity. After reperfusion injury, Complex Ⅰ function and activity were impaired in steatotic livers compared to lean livers. Ischemic preconditioning did not influence mitochondrial function in this setting.

Terminology

Mitochondrial Complex Ⅰ is paramount for intact mitochondrial function, and impairment of Complex Ⅰ leads to impaired ATP (cellular energy currency) production and consequently cell death.

Peer-review

It is well known that hepatic steatosis increases susceptibility to IRI and that this effect is linked to mitochondrial dysfunction. This study explored alterations of mitochondrial Complexes Ⅰ and Ⅱ in lean and high-fat, high sucrose diet–induced steatotic rat livers after 1 h-warm ischemia plus 4 h reperfusion. The authors showed that there was a significant decrease in Complex Ⅰ in steatotic livers compared to lean livers but there was no difference in Complex Ⅱ between these two groups. IPC decreased alanine aminotransferase release and histological changes after IRI but did not blunt decreases in Complex Ⅰ in steatotic livers. This study obtained some interesting data.

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ORIGINAL ARTICLE

Basic Study

Contribution of mammalian target of rapamycin in the pathophysiology of cirrhotic cardiomyopathy

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Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at dehpour@yahoo.com. No additional data are available.

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Abstract

AIM: To explore the role of mammalian target of rapamycin (mTOR) in the pathogenesis of cirrhotic cardiomyopathy and the potential of rapamycin to improve this pathologic condition.

METHODS: Male albino Wistar rats weighing 100-120 g were treated with tetrachloride carbon (CCl_4) for 8 wk to induce cirrhosis. Subsequently, animals were administered rapamycin (2 mg/kg per day). The QT_c intervals were calculated in a 5-min electrocardiogram. Then, the left ventricular papillary muscles were

isolated to examine inotropic responsiveness to β-adrenergic stimulation using a standard organ bath equipped by Powerlab system. Phosphorylated-mTOR localization in left ventricles was immunohistochemically assessed, and ventricular tumor necrosis factor (TNF)-α was measured. Western blot was used to measure levels of ventricular phosphorylated-mTOR protein.

RESULTS: Cirrhosis was confirmed by hematoxylin and eosin staining of liver tissues, visual observation of lethargy, weight loss, jaundice, brown urine, ascites, liver stiffness, and a significant increase of spleen weight ($P < 0.001$). A significant prolongation in QT_c intervals occurred in cirrhotic rats exposed to CCl_4 ($P < 0.001$), while this prolongation was decreased with rapamycin treatment ($P < 0.01$). CCl_4-induced cirrhosis caused a significant decrease of contractile responsiveness to isoproterenol stimulation and a significant increase in cardiac TNF-α. These findings were correlated with data from western blot and immunohistochemical studies on phosphorylated-mTOR expression in left ventricles. Phosphorylated-mTOR was significantly enhanced in cirrhotic rats, especially in the endothelium, compared to controls. Rapamycin treatment significantly increased contractile force and myocardial localization of phosphorylated-mTOR and decreased cardiac TNF-α concentration compared to cirrhotic rats with no treatment.

CONCLUSION: In this study, we demonstrated a potential role for cardiac mTOR in the pathophysiology of cirrhotic cardiomyopathy. Rapamycin normalized the inotropic effect and altered phosphorylated-mTOR expression and myocardial localization in cirrhotic rats.

Key words: Cirrhotic cardiomyopathy; Rat; Mammalian target of rapamycin; Rapamycin; Inotropic effect

Core tip: Enhanced levels of cardiac phosphorylated mammalian target of rapamycin (mTOR) contribute to impairment of electrophysiological and mechanical function induced by cirrhosis, called "cirrhotic cardiomyopathy". Here, we find that the mTOR inhibitor rapamycin normalized the impaired inotropic responsiveness to β-adrenergic stimulation and prolonged Q-T interval in tetrachloride carbon (CCl_4)-induced cirrhotic rats. Cardiac ventricular expression of phosphorylated-mTOR (p-mTOR) was increased in rats with cirrhosis, and this effect was ameliorated by rapamycin. CCl_4-induced cirrhosis was associated with an increase in cardiac proinflammatory cytokine tumor necrosis factor-α, and this increase was reversed by rapamycin as well.

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INTRODUCTION

For a long time, cardiac dysfunction in liver cirrhosis, termed "cirrhotic cardiomyopathy", was thought to be a common occurrence in patients suffering from alcoholic cirrhosis[1,2]. During the last decade, however, non-alcoholic cirrhotic patients have also been reported to demonstrate these cardiac abnormalities[3]. Cardiovascular dysfunction is observed in cirrhosis, but the underlying mechanisms are not still well understood. Despite the hyperdynamic systemic circulation and the absence of coronary artery or valvular disease and hypertension, cardiac hypertrophy and cardiomyocyte edema are observed in cirrhotic patients[3-7]. Furthermore, there is evidence for a concomitant decrease of inotropic effect along with impaired myocardial contractility[6]. Previous studies have shown that both portal hypertension and cirrhosis contribute to cardiomyopathy[1,8]. Cardiomyopathy is characterized by latent heart failure with impaired contractile responsiveness to pharmacological or physiological stress and/or altered diastolic relaxation with electrophysiological abnormalities, without any diagnosed cardiac disease and causes of cirrhosis[4,6].

A variety of mechanisms are responsible for the pathogenesis of cirrhotic cardiomyopathy. The major predisposing factors of cardiac contractility include alteration in ventricular receptor signal transduction (*i.e.*, β-adrenergic, muscarinic, and cannabinoid receptors)[9-12] and ionic channel function (*i.e.*, K^+ and L-type voltage-gated Ca^{2+})[13-15], cardiomyocyte plasma membrane fluidity changes[5,6], and complex alterations of carbon monoxide and nitric oxide (NO)[16,17]. Moreover, a rise in pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) is observed in this condition, resulting in stimulation of inducible nitric oxide synthase (iNOS) and NO overproduction[18].

Mammalian target of rapamycin (mTOR), a serine/threonine kinase component downstream of phosphotidylinositide 3-kinase (PI3K)/Akt signaling pathway[19,20], is a key regulator of mRNA translation and cell growth in cardiomyocytes[21,22]. Protein synthesis, a major factor for cardiac hypertrophic growth, is regulated by the PI3K/Akt/mTOR signaling pathway through inactivation of eukaryotic translation initiation factor 4E-binding proteins (4E-BPs)[23], leading to stimulation of polymerase Ⅰ and Ⅲ transcription[24], control of ribosome biogenesis and mitochondrial metabolism[25], and suppression of autophagy[26-28]. Zhang *et al*[29] found that mTOR knockout mice had improved baseline cardiomyocyte survival, decreased dilated cardiac hypertrophy, and less heart failure than control mice. Moreover, it was shown that activation of PI3K/Akt/mTOR signaling may lead to the development of cardiac

hypertrophy[30]. Indeed, the mTOR inhibitor rapamycin appeared to block the development of cardiomyocyte hypertrophy[29], and cohort studies have shown that rapamycin has cardioprotective effects in patients after liver transplantation[31,32].

Although our current knowledge of the predisposing factors of cirrhotic cardiomyopathy is somewhat understood, the role of other pathophysiological mechanisms underlying cardiac dysfunction induced by cirrhosis remains to be clarified. To this purpose, we examined the hypothesis that tetrachloride carbon (CCl_4)-induced cardiac inotropic dysfunction in response to adrenergic stimulation is associated with altered expression of cardiac p-mTOR in a rat model of cirrhotic cardiomyopathy. In this study, we demonstrate for the first time the positive inotropic effect of mTOR suppression by rapamycin and its ability to normalize cardiac levels of p-mTOR and the pro-inflammatory factor TNF-α in cirrhotic cardiomyopathy.

MATERIALS AND METHODS

Chemicals and reagents

The following compounds and reagents were applied in this investigation: rapamycin (Wyeth, Kildare, United Kingdom/Ireland), isoproterenol hydrochloride (Sigma, St. Louis, MO, United States), carbon tetrachloride (Merck, Darmstadt, Germany); TNF-α assay kit, polyclonal p-mTOR antibody (pSer2448), and horseradish peroxidase (HRP)-conjugated rabbit anti-rat Immunoglobulin G antibody (Biorbyt Co. Ltd., Cambridge, United Kingdom).

Animal model of cirrhosis

Male albino Wistar rats weighing 100-120 g were used with housing facilities (environment temperature at 21 ℃-23 ℃, 12-h regular light/dark cycle). Animals had unlimited access to food and water except for a brief time during injection and during the surgical procedure. The rats were divided into four main groups: control/drinking water, control/rapamycin, cirrhotic/drinking water, and cirrhotic/rapamycin. All experiments and manipulations were conducted in Prof. Dehpour's Hepatological Research Laboratory in accordance with the institutional animal care and use committee (Department of Pharmacology, School of Medicine, Tehran University of Medical Sciences) guidelines. This study was approved by the Ethics Committee of Tehran University of Medical Sciences.

To induce cirrhosis, CCl_4 (0.4 g/kg; a solution of 1:6 in mineral oil) was intraperitoneally injected to the animals three times a week for 8 wks until the appearance of ascites[33]. Rapamycin (2 mg/kg per day) was freshly dissolved in normal saline and daily administered in drinking water in a constant volume of 14 mL/100 g body weight during the 8-wk period[34,35]. Twenty-four hours after cessation of CCl_4, animals were sacrificed by guillotine decapitation. The liver was removed, sectioned, and stained with hematoxylin-eosin (HE). Light microscopy of stained liver sections confirmed the induction of cirrhosis in rats[4].

Twenty-four hours after the last administration of either CCl_4 or N/S, a lead Ⅱ electrocardiogram (ECG) was recorded for 15 min using three stainless steel subcutaneous electrodes attached to a bioamplifier (ADInstrument, Sydney, Australia) from the anesthetized rats. The signals were digitized at a sampling rate of 10 kHz by a Powerlab system and were displayed using Lab Chart 7 software (ADInstrument). The Q-T intervals, presented as corrected Q-T (QT_c), were calculated in a 5-min ECG. The QT_c was presented using Bazett's formula ($QT_c = QT/\sqrt{R\text{-}R}$)[36].

Preparation of isolated papillary muscle

Briefly, animals' hearts were excised following decapitation and left ventricular papillary muscles were dissected in cold oxygenated physiological salt solution (PSS) containing (in mmol/L) NaCl, 112; KCl, 5; $CaCl_2$, 1.8; $MgCl_2$, 1; NaH_2PO_4, 0.5; KH_2PO_4, 0.5; $NaHCO_3$, 25; glucose, 10; and EDTA, 0.004[37,38]. The isolated papillary muscles were suspended in a 25-mL organ bath chamber containing PSS buffer solution bubbled with a gas mixture of 95% O_2: 5% CO_2 at 37 ℃ for 90 min to reach equilibrium. The contractility was induced by electrical field stimulation (Grass 88 Stimulator; Grass Instruments, West Warwick, RI, United States) at 1 Hz and 30 V, 20% higher than the threshold. After achievement of baseline contractile force, the muscle contraction was stimulated by addition of cumulative concentrations of isoproterenol (10^{-10} to 10^{-5} mol/L). The contractile force induced by the highest concentration of isoproterenol (10^{-5} mol/L) was considered as maximal contractility[16]. The resulted contractile forces were expressed as a percentage of the baseline papillary muscle contractility.

Immunohistochemistry

The ventricle samples were immediately fixed in freshly prepared 10% formalin and paraffin-embedded blocks. After deparaffinizing in xylene and rehydrating in decreasing concentrations of ethanol, 3% hydrogen peroxidase was added for 5 min to block dual endogenous peroxidase activity. Then, the immunohistochemical staining was performed based on the Avidin-Biotin peroxidase method. Polyclonal p-mTOR antibody (pSer2448) (1:50 dilution) was reacted for 1 h at room temperature followed by secondary HRP-conjugated rabbit anti-rat Immunoglobulin G antibody (1:50 dilution) for 30 min at room temperature. The sections were washed three times with Tris (pH 7.4), incubated with diaminobenzidine (DAB) solution for 10 min, and then incubated with 5% $CuSO_4$ for 5 min. Ultimately, the slides were washed and counterstained with H&E to obtain brown-colored precipitation for examination under light microscopy.

Ventricular TNF-α quantification

To measure tissue TNF-α, the left ventricles were excised, rinsed in PSS, snap-frozen in liquid nitrogen, and stored at -80 ℃ for further analysis. The samples were then homogenized in ice-cold phosphate-buffered saline (PBS) and centrifuged at 14200 *g* for 30 min. Fifty microliters of the samples and standards were pipetted into a 96-well plate precoated with rat TNF-α specific antibody. Following addition of 50 μL of biotinylated anti-TNF-α solution, the plate of the enzyme linked immunosorbent assay kit was incubated for 90 min at room temperature. The wells were washed, exposed to 100 μL of streptavidin-peroxidase, incubated for 45 min at room temperature, and washed four times with PBS. Finally, 100 μL of both stabilized chromogen and stop solution were respectively added in two stages and incubated for 20 min for spectrophotometrically analysis at λ = 450 nm[16].

Western blot analysis

The dissection and snap-freezing of left ventricles were performed as described in the above section. Briefly, left ventricles were homogenized in buffer (20 mmol/L Tris-HCl (pH 7.2), 0.2 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L dithiothreitol), centrifuged at 40000 *g*, and resuspended in Tris buffer containing proteinase inhibitor. Thirty micrograms of protein samples were loaded and separated on sodium dodecyl sulfate-10% polyacrylamide gel (SDS-PAGE) by electrophoresis and were wet electroblotted onto nitrocellulose membrane at 4 ℃ for 12 h[39,40]. The blots were blocked for 1 h at room temperature with 2% bovine serum albumin in 0.1% Tween Tris-buffered saline (TBS-T) (pH 7.5). Then, the membranes were washed and incubated overnight at 4 ℃ with polyclonal p-mTOR primary antibody (pSer2448) (1:100 dilution). After washing, these blots were exposed to HRP-conjugated anti-rat secondary antibody (1:1000 dilution). Detection of blots was performed using enhanced chemiluminescence (ECL kit, Amersham, Chalfont St. Giles, United Kingdom) method. The levels of p-mTOR in cirrhotic, control, and rapamycin-treated animals were semi-quantified using ImageJ software (National Institutes of Health, Bethesda, MD, United States), which was defined as the p-mTOR/glyceraldehyde 3 phosphate dehydrogenase (GAPDH) densitometric ratio (%).

Statistical analysis

All data are expressed as mean ± SD and analyzed using GraphPad Prism software (version 5.0, GraphPad Software, Inc., La Jolla, CA, United States). To examine the differences between three or more experimental groups, one-way analysis of variance (ANOVA) followed by a Tukey's post test was used. For two-group comparisons, Student's *t*-test was applied. Evaluation of the effects of two variables (cirrhosis *vs* control and type of treatment) was performed using two-way ANOVA followed by a Bonferroni post test. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Presence of CCl_4-induced cirrhosis was confirmed by visual observation of lethargy, weight loss, jaundice, brown urine, and ascites along with liver stiffness and a significant increase in spleen weight (1.52 ± 0.13 g *vs* 2.74 ± 0.41 g in control *vs* cirrhotic rats, $P < 0.001$), which contributed to the development of portal hypertension. H&E staining of liver tissues sampled from cirrhotic rats demonstrated focal hepatocellular necrosis and apoptotic cells as well as enhanced inflammatory cell infiltration into the portal tract. Fatty degeneration areas and central vein dilation were also seen histologically (Figure 1). Moreover, cirrhosis model animals had significantly prolonged QT_c intervals compared to controls ($P < 0.001$; Figure 2). The prolonged QT_c interval in cirrhotic rats was decreased by rapamycin (2 mg/kg) ($P < 0.01$; Figure 2).

Effect of rapamycin on papillary muscle contractility

As shown in Figure 3A, baseline papillary muscle inotropic responses to isoproterenol stimulation in cirrhotic rats were significantly decreased compared to controls ($P < 0.001$). The order was in agreement with the maximum response (R_{max}) to isoproterenol (76.46% ± 10.08% *vs* 117.36% ± 8.25%, $P < 0.001$; Figure 3A). Rapamycin did not significantly alter R_{max} in control rats. Likewise there was no significant difference in the EC_{50} of isoproterenol ($4.08 \pm 1.35 \times 10^{-8}$ and $6.59 \pm 1.29 \times 10^{-8}$ in N/S- and rapamycin-treated non-cirrhotic control groups, respectively; $P > 0.05$; Figure 3B). In cirrhotic rats, there was a significant rise in papillary muscle contractility and a significant enhancement of R_{max} following chronic treatment with rapamycin (2 mg/kg) compared to cirrhotic rats treated with N/S ($P < 0.001$; Figure 3C). There were no significant differences in the EC_{50} of isoproterenol among all four studied groups ($P > 0.05$; Figure 3D).

Effect of rapamycin treatment on ventricular TNF-α concentration

As shown in Figure 4, there was a significant increase in ventricular levels of TNF-α in cirrhotic rats compared to controls ($P < 0.001$). Treatment with rapamycin (2 mg/kg) for 8 wk caused no marked enhancement in tissue TNF-α concentration in the control group ($P > 0.05$). In addition, rapamycin significantly decreased the elevation in tissue TNF-α concentration in animals with cirrhosis ($P < 0.05$).

Ventricular p-mTOR expression

As shown in Figure 5, expression of p-mTOR in the left

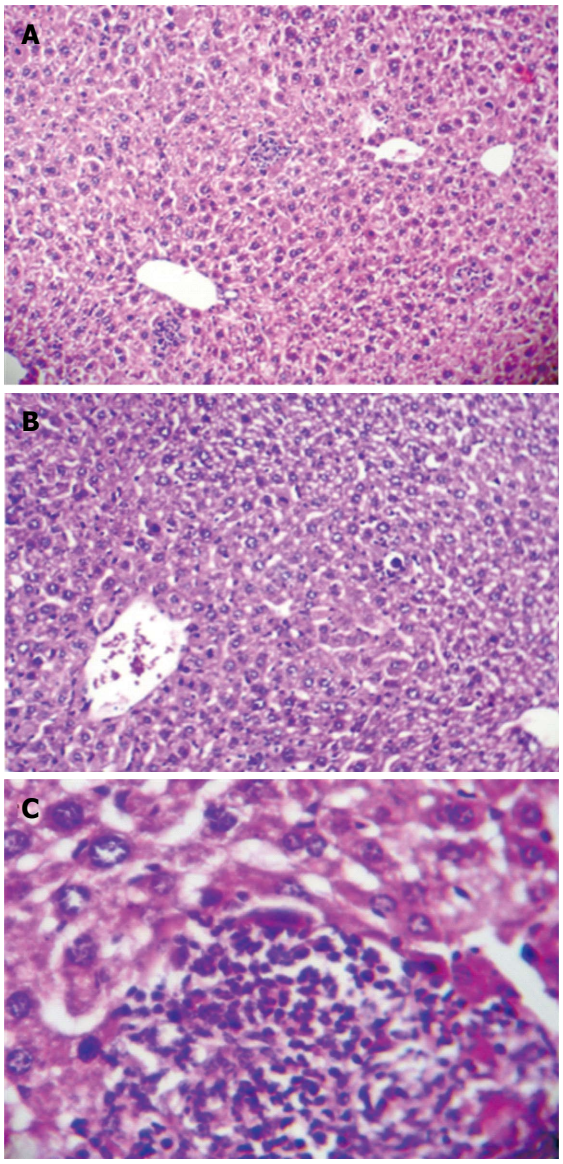

Figure 1 Histological change in liver tissue of CCl_4-induced cirrhotic rats (hematoxylin and eosin; magnification × 100 and × 400). A: Focal hepatocellular necrosis, apoptotic cells, and patchy inflammatory cell infiltration along with central vein dilation are observed; B: Fatty degeneration areas are clearly seen; C: Inflammatory cell infiltration into the portal tract.

ventricles of cirrhotic rats was increased compared to controls ($P < 0.001$). Rapamycin treatment reversed this increase in p-mTOR level in animals with cirrhosis ($P < 0.001$). Moreover, treatment of cirrhotic rats with rapamycin decreased p-mTOR protein expression to the level of control animals ($P > 0.05$).

To explore which cells express p-mTOR, immunohistochemical analysis was performed. Although almost no immunostaining was observed in the ventricular myocytes and endothelial cells in the control group (Figure 6A), p-mTOR immunostaining was markedly stronger in endothelial cells, but not in myocardial layer, of cirrhotic rats (Figure 6B). In the cirrhosis group, rapamycin could decrease p-mTOR immunostaining and induce mTOR phosphorylation in ventricular myocytes, as shown in Figure 6D.

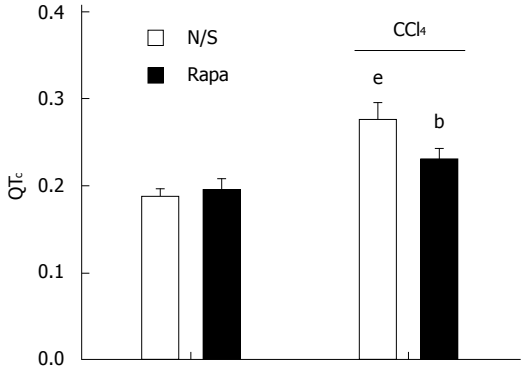

Figure 2 QT interval in control and CCl_4-induced cirrhotic rats treated with normal saline or rapamycin (2 mg/kg). QT intervals were defined as corrected QT (QT_c) using Bazett's formula. The data are expressed as the mean ± SD. $^eP < 0.001$ *vs* control/normal saline group; $^bP < 0.01$ *vs* control/rapamycin and cirrhotic/saline group.

DISCUSSION

The main finding of the present study is the demonstration that cardiac mTOR expression and protein levels are increased in rats with cirrhotic cardiomyopathy. For the first time we showed that altered expression of p-mTOR in cirrhotic heart contributed to cardiac contractile suppression. This effect was confirmed by immunohistochemical assay, which showed a strong p-mTOR signal in cirrhotic left ventricles, especially in endothelial cells. Interestingly, the data from an *in vitro* papillary muscle study suggested that the enhanced expression of p-mTOR caused cardiac dysfunction. Consistent with that finding, we found a relationship between changes of mTOR activity and hypertrophic cardiomyopathy and heart failure[20,30,40-44]. Moreover, an increase in cardiac tissue TNF-α was observed in cirrhotic animals, which was accompanied by cardiomyocyte contractile dysfunction. Recently, several studies have investigated the role of TNF-α in the pathogenesis of heart failure and impaired cardiac contractility and have demonstrated that increased NO synthesis, an underlying mechanism for cirrhosis, in cardiac tissues of cirrhotic mice is attributed to elevated TNF-α level[4].

We also showed that repeated treatment with rapamycin normalized the cardiac contractile force defect in cirrhotic rats. To our knowledge, this is the first investigation to examine the hypothesis that rapamycin, *via* mTOR suppression, improves cardiac inotropic responsiveness to isoproterenol β-adrenergic stimulation and shortens the prolonged QT_c in rats with cirrhosis. Since mTOR phosphorylation was not obviously detectable in ventricular cardiomyocytes taken from CCl_4-induced cirrhotic rats, rapamycin caused significantly greater increases in p-mTOR protein level in cardiomyocytes than endothelial cells. Interestingly, despite the abundant expression of p-mTOR in cardiomyocytes, but not in endothelial cells, of rapamycin-treated rats with cirrhosis, total

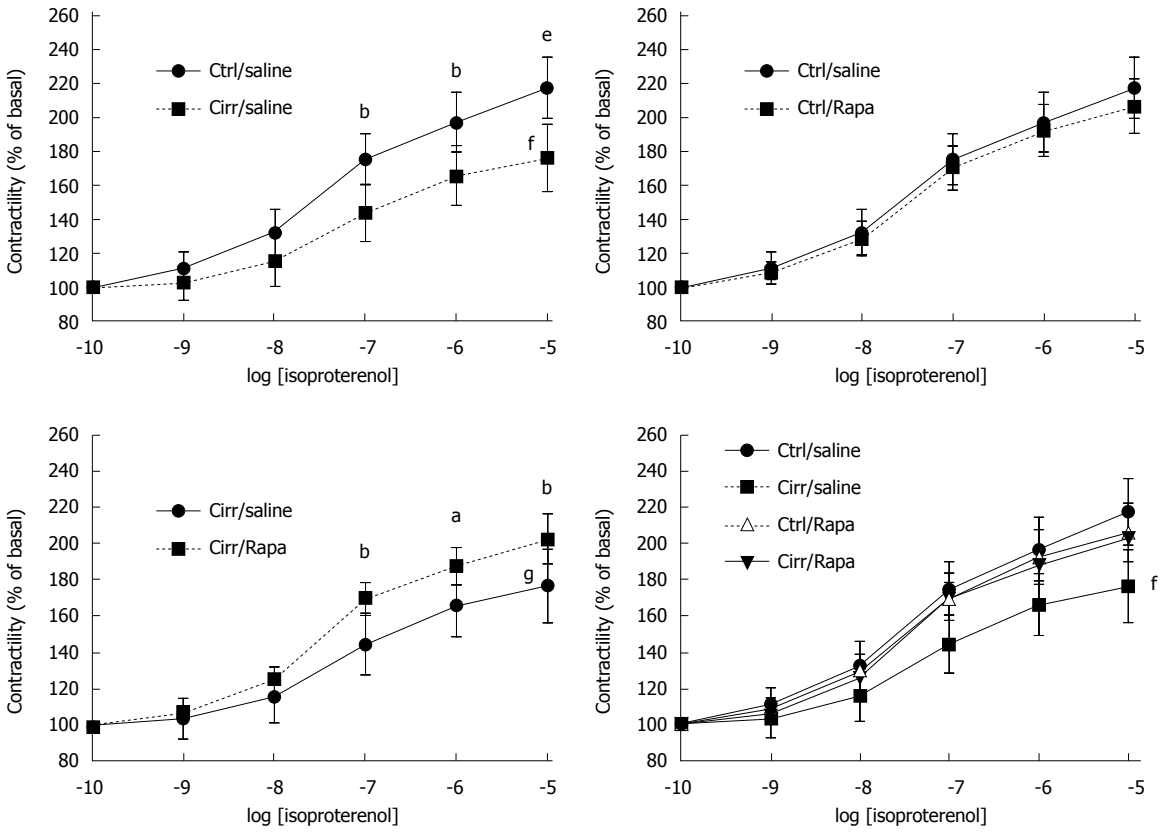

Figure 3 Contractile force in response to β-adrenergic stimulation in cirrhotic and control rats treated with normal saline or rapamycin (2 mg/kg). Inotropic responsiveness to β-adrenergic stimulation with isoproterenol in the isolated papillary muscle from cirrhotic and control rats treated with normal saline or rapamycin (2 mg/kg) was analyzed to determine the contractile force (% of basal). The data are expressed as the mean ± SD. Maximal response (R_{max}) in the CCl_4-induced cirrhotic rats was significantly lower than the other groups. There were no significant differences in EC_{50} values among the four studied groups. $^{f}P < 0.001$ *vs* the control group receiving normal saline; $^{g}P < 0.001$ *vs* the cirrhosis group receiving normal saline; $^{a}P < 0.05$, $^{b}P < 0.01$, $^{e}P < 0.001$ *vs* the cirrhotic group receiving normal saline in that concentration.

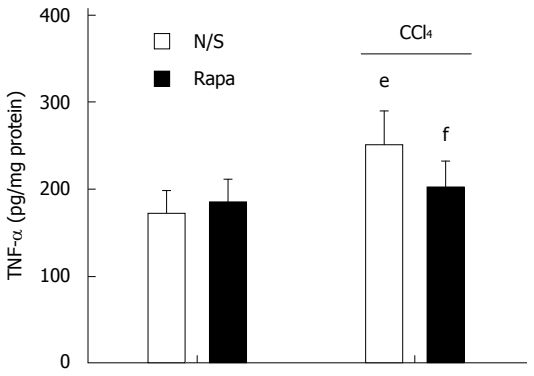

Figure 4 Left ventricular tumor necrosis factor-α levels in control and CCl_4-induced cirrhotic rats treated with normal saline or rapamycin (2 mg/kg). The data are expressed as the mean ± SD. $^{e}P < 0.001$ *vs* control/normal saline group; $^{f}P < 0.001$ *vs* cirrhosis/normal saline group. TNF-α: Tumor necrosis factor-α.

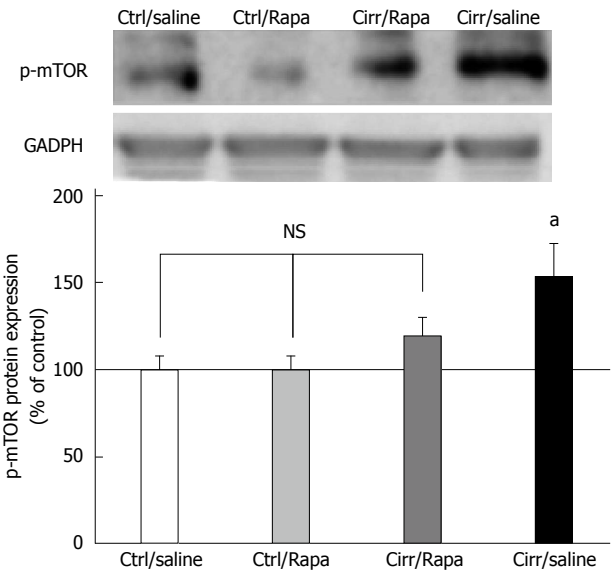

Figure 5 Western blot analysis of p-mammalian target of rapamycin protein in the left ventricles of control and CCl_4-induced cirrhotic rats treated with normal saline or rapamycin (2 mg/kg). The upper panels demonstrate the representative immunoblots of p-mTOR and glyceraldehyde 3 phosphate dehydrogenase (GAPDH) proteins in the control, control + rapamycin, cirrhotic and cirrhotic + rapamycin. The lower panel shows the densitometric analysis after normalization with GAPDH. Values are expressed as p-mTOR/GAPDH ratio (%) and normalized to the control group receiving normal saline (mean ± SD). $^{a}P < 0.05$ *vs* the other three groups; NS: Non-significant.

cardiac p-mTOR protein was reduced in comparison with cirrhotic rats receiving N/S. This finding was correlated with the positive inotropic effects of rapamycin in this paradigm. Decreased tissue levels of TNF-α after treatment with rapamycin confirmed the hypothesis that reduction in overproduced cytokines, such as TNF-α and interleukin-1β, from hepatic and systemic reticuloendothelial cells can reverse

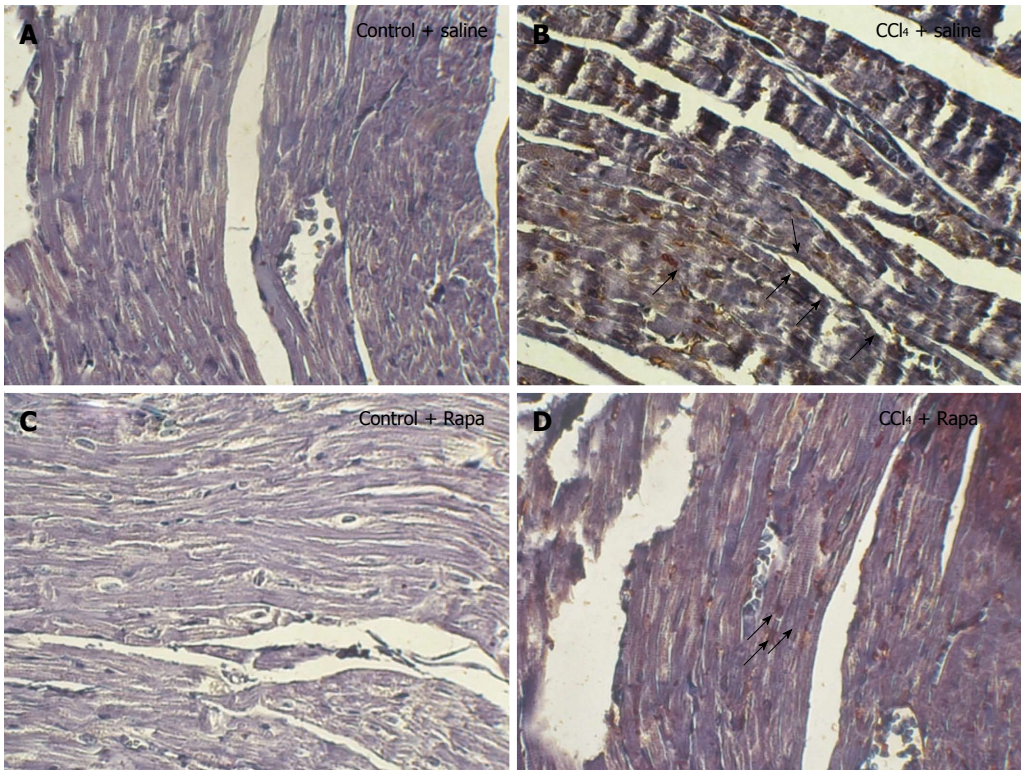

Figure 6 Immunohistochemical staining for p-mTOR in the ventricles of the rats in the following groups: control, cirrhotic, control + rapamycin and cirrhotic + rapamycin (× 400 magnification). Human gastric tissue was used as the positive control. Note the increased immunostaining of p-mTOR in the myocytes of the rats with cirrhosis. No significant immunostaining was localized to the cardiomyocytes of the untreated cirrhotic rats. In contrast, treatment with rapamycin caused significant immunostaining in the cardiomyocytes of the cirrhotic rats. The black arrows indicate to the p-mTOR immunoblots in rat ventricles.

their negative inotropic effects[16,45,46]. Evidence has shown that rapamycin acts as an effective agent, like isoproterenol, to raise intracellular cyclic adenosine monophosphate by reducing the expression and release of the pro-inflammatory cytokine TNF-α from human heart tissue[47]. Also, rapamycin may inhibit nuclear factor-kappa B (NFκB) activation and TNF-α, a potent inducer of in vascular smooth muscles[48].

During the last two decades, many investigations have been performed to explore the possible manifestations and potential mechanisms underlying cirrhotic cardiomyopathy. For instance, a decrease in isolated papillary muscle contractile force was observed in response to adrenergic stimulation in bile duct-ligated rats[12,36,49-51]. These results were similar to our observation that negative inotropic responsiveness to adrenergic stimulation is a result of CCl_4-induced cirrhosis. Although most of the studies are based on the hypothesis that defects of cardiac contractile force may result from downregulation of β-adrenergic receptors[10,37] as well as increased cardiac NO synthesis[16], we tried to investigate the role of mTOR inhibition in a rat model of cirrhosis to attenuate the impairment in cardiac contractile performance. Previous studies have reported the protective effects of rapamycin on the development of left ventricle hypertrophy and ischemia/reperfusion injury after myocardial infarction[21,22,42-44,52]. Blockade of NFκB and PI3K/Akt/mTOR signaling pathway may play an essential role in ameliorating myocardial hypertrophy induced by p70S6K, a main component downstream of mTOR, activation in the infarcted hearts[21,22,30,43]. In addition to the role of mTOR in cardiomyocyte hypertrophy, p-mTOR played a role in the impairment of cardiac survival and structure and also myocardial contractile dysfunction[53]. Inhibition of mTOR activated 4E-BP1, another downstream target of mTOR, resulting in inhibition of protein synthesis, pathogenesis of cardiomyopathy, and subsequent complications of cardiac hypertrophy[29,43].

Moreover, increment of autophagy and autophagosome formation upon mTOR inhibition with rapamycin is considered to be other protective mechanisms in heart failure[43,54]. Regarding the requirement of the ubiquitin proteasome system for activation of NFκB, rapamycin can restrict the myocardial infarction size and remodeling by inhibiting the ubiquitin proteasome and subsequent NFκB activity[43,55,56].

In addition to the observed positive effect of rapamycin on electrophysiological and mechanical cardiac function in cirrhosis, it is noteworthy that rapamycin has protective effects on human liver fibrosis and inhibits the progression of fibrosis, especially at early stages[35,57,58]. Rapamycin exerts this effect by inhibiting cell proliferation, deposition of extracellular matrix, and the profibrogenic pathway and factors[59-62]. Additionally, cohort studies have reported that patients receiving rapamycin after liver

transplantation had no cardiovascular problems. They showed that rapamycin not only did not increase the risk of congestive heart failure and myocardial infarction but plays a role as a cardioprotective agent[31,32]. In our study, the positive role of rapamycin on cirrhotic cardiomyopathy was attributed to a direct effect on cirrhotic heart, and it was assumed that a part of this phenomenon was associated with the anti-fibrogenic effect of this drug. This assumption is strongly amplified since cardiac and liver diseases share a common etiology[6]. Although experimental and clinical investigations on cirrhotic patients revealed latent heart failure with impaired response to provocations and subsequent mortality, no effective treatment has been found for improving cardiac contractility in patients with cirrhotic cardiomyopathy and evident ventricular failure[6]. As the prolongation in QT interval is considered an important life-threatening element in patients with cirrhotic cardiomyopathy, early identification and treatment of patients are necessary. Therefore, due to the anti-cytokine and beneficial role of rapamycin in correcting the abnormal cardiac contractile force and QT interval, rapamycin is expected to be the subject for further clinical investigations in patients with cirrhotic cardiomyopathy.

The present study has provided evidence that an increase in p-mTOR is responsible for the impaired cardiac contractility in animals with CCl_4-induced cirrhosis. Moreover, mTOR blockade corrected the cardiac contractile dysfunction in liver cirrhosis, highlighting the possible therapeutic potential for the mTOR antagonist rapamycin in this condition. This treatment may increase survival in cirrhosis-associated heart failure until a transplant becomes available. In addition, our utilization of an experimental model of cirrhotic cardiomyopathy and its translation to clinical benefits may guide future research studies.

COMMENTS

Background

"Cirrhotic cardiomyopathy" has been recognized as cardiac dysfunction in liver cirrhosis, which commonly occurs in patients suffering from cirrhosis. Unfortunately, the responsible mechanisms underlying the pathophysiology of cirrhotic cardiomyopathy are not well understood. Therefore, understanding these mechanisms may help to develop possible treatments for this disease.

Research frontiers

To date, a variety of mechanisms have been described that are responsible for the pathogenesis of cirrhotic cardiomyopathy. The major predisposing factors of cardiac contractility include alterations in ventricular receptor signal transduction and ionic function, cardiomyocyte plasma membrane fluidity changes, and complex alterations in carbon monoxide and nitric oxide.

Innovations and breakthroughs

Although the current knowledge of the mechanisms underlying cirrhotic cardiomyopathy is somewhat understood, the role of other pathophysiological mechanisms remains to be clarified. To this purpose, the authors examined the hypothesis that CCl_4-induced cardiac inotropic dysfunction in response to adrenergic stimulation is associated with altered expression of cardiac phosphorylated-mammalian target of rapamycin (mTOR) in a rat model of cirrhotic cardiomyopathy. Therefore, this study is the first to demonstrate the positive inotropic effect of mTOR suppression by rapamycin and its ability to normalize cardiac levels of phosphorylated-mTOR as well as the pro-inflammatory factor TNF-α in cirrhotic cardiomyopathy.

Applications

mTOR blockade corrected the cardiac contractile dysfunction in liver cirrhosis, highlighting the therapeutic potential of the mTOR antagonist rapamycin in this condition. Treatment with rapamycin may increase survival in those with cirrhosis-associated heart failure until a transplant becomes available. This study may guide researches to utilize the experimental model of cirrhotic cardiomyopathy translating to clinical benefits.

Peer-review

This is an interesting study about the role of mTOR in the pathogenesis of cirrhotic cardiomyopathy and the potential use of rapamycin for improving cardiac dysfunction.

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ORIGINAL ARTICLE

Basic Study

Qinggan Huoxue Recipe suppresses epithelial-to-mesenchymal transition in alcoholic liver fibrosis through TGF-β1/Smad signaling pathway

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Abstract

AIM: To investigate the mechanism by which Qinggan Huoxue Recipe (QGHXR) inhibits epithelial-to-mesenchymal transition (EMT) in rats with alcoholic liver fibrosis (ALF).

METHODS: A total of 75 male SD rats were used to induce ALF. Serum biochemical indicators, including

alanine aminotransferase, aspartate aminotransferase, laminin and hyaluronidase, were measured. Liver histopathological changes were evaluated using hematoxylin-eosin and Sirius red staining. EMT was examined by analyzing the expression of the epithelial marker E-cadherin and the mesenchymal markers vimentin and fibronectin using RT-PCR and Western blot. The inhibitory effect of QGHXR on EMT markers, as well as its effect on molecules associated with the transforming growth factor (TGF)-β1/Smad signaling pathway, including TGF-β1, Smad3, snail, occludin, ZO-1 and claudin, was also examined.

RESULTS: Compared with normal control rats, ALF rats exhibited a decrease in E-cadherin levels (mRNA: ALF 0.16 ± 0.05 *vs* control 1.00 ± 0.08; protein: ALF 0.09 ± 0.05 *vs* control 0.70 ± 0.17, $P < 0.01$) and an increase in vimentin and fibronectin levels (mRNA: 11.43 ± 0.39 *vs* 1.00 ± 0.19 and 9.91 ± 0.34 *vs* 1.00 ± 0.44, respectively, $P < 0.01$; protein: 1.13 ± 0.42 *vs* 0.09 ± 0.03 and 1.16 ± 0.43 *vs* 0.09 ± 0.00, respectively, $P < 0.01$). This indicates that EMT occurred in ALF rats. In addition, the TGF-β1/Smad signaling pathway was activated in ALF rats, as evidenced by the increase in TGF-β1 and snail levels (mRNA: 1.76 ± 0.12 *vs* 1.00 ± 0.05 and 6.98 ± 0.41 *vs* 1.00 ± 0.10, respectively, $P < 0.01$; protein: 1.43 ± 0.05 *vs* 0.12 ± 0.03 and 1.07 ± 0.29 *vs* 0.07 ± 0.02, respectively, $P < 0.01$) and the decrease in Smad3 levels (mRNA: 0.05 ± 0.01 *vs* 1.00 ± 0.12, $P < 0.01$; protein: 0.06 ± 0.05 *vs* 0.89 ± 0.12, $P < 0.01$). Furthermore, levels of the tight junction markers occludin, ZO-1 and claudin decreased in ALF rats compared with healthy control rats (mRNA: 0.60 ± 0.09 *vs* 1.00 ± 0.12, 0.11 ± 0.00 *vs* 1.00 ± 0.12 and 0.60 ± 0.01 *vs* 1.00 ± 0.08, respectively, $P < 0.01$; protein: 0.05 ± 0.01 *vs* 0.87 ± 0.40, 0.09 ± 0.05 *vs* 0.89 ± 0.18 and 0.04 ± 0.03 *vs* 0.95 ± 0.21, respectively, $P < 0.01$). In ALF rats treated with QGHXR, E-cadherin levels increased (mRNA: QGHXR 0.67 ± 0.04 *vs* ALF model 0.16 ± 0.05, $P < 0.01$; protein: QGHXR 0.66 ± 0.21 *vs* ALF model 0.09 ± 0.05, $P < 0.01$), and vimentin and fibronectin levels decreased (mRNA: 6.57 ± 1.05 *vs* 11.43 ± 0.39 and 1.45 ± 1.51 *vs* 9.91 ± 0.34, respectively, $P < 0.01$; protein: 0.09 ± 0.03 *vs* 1.13 ± 0.42 and 0.10 ± 0.01 *vs* 1.16 ± 0.43, respectively, $P < 0.01$). In addition, QGHXR inhibited the expression of TGF-β1 and increased the expression of Smad3 (mRNA: 1.03 ± 0.11 *vs* 1.76 ± 0.12, 0.70 ± 0.10 *vs* 0.05 ± 0.01, respectively, $P < 0.05$ and $P < 0.01$; protein: 0.12 ± 0.03 *vs* 1.43 ± 0.05 and 0.88 ± 0.20 *vs* 0.06 ± 0.05, respectively, $P < 0.01$). QGHXR treatment also reduced the levels of the EMT-inducing transcription factor snail (mRNA: 2.28 ± 0.33 *vs* 6.98 ± 0.41, $P < 0.01$; protein: 0.08 ± 0.02 *vs* 1.07 ± 0.29, $P < 0.01$) and increased the occludin, ZO-1 and claudin levels (mRNA: 0.73 ± 0.05 *vs* 0.60 ± 0.09, 0.57 ± 0.04 *vs* 0.11 ± 0.00 and 0.68 ± 0.03 *vs* 0.60 ± 0.01, respectively, $P < 0.01$, $P < 0.01$ and $P < 0.05$; protein: 0.92 ± 0.50 *vs* 0.05 ± 0.01, 0.94 ± 0.22 *vs* 0.09 ± 0.05 and 0.94 ± 0.29 *vs* 0.04 ± 0.03, respectively, $P < 0.01$). The effects of QGR and HXR on the TGF-β1/Smad signaling pathway were similar to that of QGHXR; however, the QGR- and HXR-induced changes in vimentin mRNA levels, the QGR-induced changes in fibronectin mRNA levels and the HXR-induced changes in snail and TGF-β1 mRNA levels were not significant.

CONCLUSION: Qinggan Huoxue Recipe inhibits EMT in ALF rats by modulating the TGF-β1/Smad signaling pathway, suggesting that the mechanism underlying the amelioration of ALF induced by QGHXR is associated with this pathway.

Key words: Alcoholic liver fibrosis; QGHXR; Epithelial-to-mesenchymal transition; Snail; Transforming growth factor-β1/Smad

Core tip: Epithelial-to-mesenchymal transition (EMT) is a dynamic process by which mature epithelial cells lose their distinct characteristics and acquire a mesenchymal phenotype. EMT is characterized by loss of the expression of the epithelial marker E-cadherin and up-regulation of the mesenchymal markers α-SMA, collagen Ⅰ, vimentin and fibronectin. Our study provided evidence that QGHXR inhibits EMT in alcoholic liver fibrosis by regulating the transforming growth factor-β1/Smad signaling pathway and that QGHXR-mediated inhibition of EMT might be a promising approach to ameliorating alcoholic liver injury.

Wu T, Chen JM, Xiao TG, Shu XB, Xu HC, Yang LL, Xing LJ, Zheng PY, Ji G. Qinggan Huoxue Recipe suppresses epithelial-to-mesenchymal transition in alcoholic liver fibrosis through TGF-β1/Smad signaling pathway. *World J Gastroenterol* 2016; 22(19): 4695-4706 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i19/4695.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i19.4695

INTRODUCTION

Alcoholic liver disease (ALD) has become a major cause of morbidity and mortality worldwide[1]. ALD progresses from a healthy liver to alcoholic steatosis, steatohepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma (HCC)[2]. Although great efforts to explore potential therapeutic targets for alcoholic liver fibrosis (ALF) have been made in recent decades, effective therapies for ALF remain an unmet need.

Epithelial-to-mesenchymal transition (EMT) is a remodeling process that occurs in adult tissues in response to pathological states, such as carcinogenesis and fibrosis[3]. EMT, a phenotypic conversion of the epithelium to a fibroblastic or myofibroblastic phenotype, plays a well-established role in the induction of fibrogenesis[4]. EMT generally begins with the dissociation of adhesions between epithelial cells, the decrease of

apical-basal polarity, the reorganization of the actin cytoskeleton and the increase of cell motility[5]. Although multiple studies have reported the significance of the EMT in fibrogenesis, the precise mechanisms underlying EMT in this context are only partially understood.

Numerous studies have demonstrated that cytokines, including transforming growth factor-β (TGF-β), epidermal growth factor (EGF) and hepatocyte growth factor (HGF), can induce EMT[6-8]. In addition, various signaling pathways associated with EMT have been found to be activated in EMT, including the TGF-β/Smad signaling pathway.

The traditional Chinese medicine formula Qinggan Huoxue Recipe (QGHXR) exerts many pharmacological effects that can ameliorate ALD, including reversing steatosis, mediating lipid peroxidation resistance and decreasing the levels of inflammatory cytokines[9,10]. A recent study demonstrated that QGHXR activated the lipopolysaccharide-Kupffer cell (LPS-KC) signaling pathway in rats with ALD by reducing serum alanine transaminase (ALT), aspartate transaminase (AST) levels and modulating CD14, NF-κB, TLR4, ERK and TNF-α expression and that QGHXR alleviated pathological changes associated with ALD[11].

However, the molecular mechanisms by which QGHXR inhibits ALD have yet to be fully elucidated. Here, we investigated the potential physiological and molecular mechanisms underlying QGHXR-mediated inhibition of EMT, especially regulation of the TGF-β/Smad signaling pathway, in an ALF rat model.

MATERIALS AND METHODS

Materials

QGHXR (bupleurum root 9 g, scutellaria root 9 g, red sage root 15 g, *Carapax trionycis* 9 g and *Radix puerariae* 15 g), Qinggan Recipe (QGR: bupleurum root 9 g and scutellaria root 9 g) and Huoxue Recipe (HXR: red sage root 15 g, *Carapax trionycis* 9 g and *Radix puerariae* 15 g), were used at concentrations of 4.75, 1.5 and 3.25 g/mL, respectively, and they were generated at the Department of Pharmacy of Longhua Hospital (Shanghai, China). Antibodies against E-cadherin, vimentin, fibronectin, TGF-β1, Smad3, snail, occludin, zona occludens-1 (ZO-1), claudin and GAPDH were purchased from Abcam (Cambridge, MA, United States). Parazole was purchased from Sigma-Aldrich Co. LLC (St Louis, MO, United States). All other chemical reagents were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

Animals and experimental design

All experiments were approved by the Local Ethics Committee for Animal Research Studies at the Shanghai University of Traditional Chinese Medicine. Male specific pathogen-free SD rats were purchased from Slac Laboratory Animal Center, Inc. (Shanghai, China). A rat ALF model was established by treating the rats with an alcohol mixture (500 mL/L alcohol, 8 mL/kg per day; pyrazole, 24 mg/kg per day; corn oil, 2 mL/kg per day) once per day, accompanied with administering intraperitoneal injections with a 25% solution of CCl_4 in olive oil (0.25 mL/kg) twice a week for 12 wk as previously described[12]. After 8 wk, the ALF rats were divided into 4 groups: control, QGR, HXR and QGHXR groups (n = 15 rats per group). Rats in the QGR, HXR, and QGHXR groups were administered a daily treatment dose of 137.5, 62.5, 200 mg/kg, respectively, by gastric lavage. An equal volume of normal saline was administered to the control ALF and healthy control groups (n = 15) by gastric lavage. The treatment lasted 4 wk. All rats were then anesthetized with 2% pentobarbital sodium (2 mL/kg) and sacrificed, and blood and liver tissue specimens were subsequently collected. One section of the liver tissue was fixed for histopathology, and another section was used for real-time polymerase chain reaction (PCR) and Western blot assays. Serum biochemical assays including measurements of ALT, AST levels were conducted using an automatic biochemistry analyzer (Hitachi Ltd, Tokyo, Japan). Serum laminin and hyaluronidase levels were detected by Shanghai Adicon Clinical laboratories Inc.

Liver histological analysis

Hematoxylin and eosin (HE) and Sirius red staining were routinely performed following standard procedures. Histological analysis was performed by HE staining of paraffin-embedded liver sections. Fibrosis was assessed in tissue sections stained with Sirius red (0.3%). Briefly, the fresh liver tissue samples were fixed in 10% formalin and embedded in paraffin. The samples were sliced into 4 μm to 5 μm sections and stained with HE staining solution or with a 0.1% Sirius red-picric solution. The latter sections were washed rapidly with acetic acid. The stained sections were observed and photographed under a light microscope at a magnification of × 200.

RNA preparation and RT-PCR analysis

Total RNA was isolated from rat liver tissues with TRIzol reagent (Invitrogen, Carlsbad, CA, United States) and reverse-transcribed into cDNA using a Reverse Transcription System (Promega, Madison, WI, United States). The thermal cycling conditions were as follows: 95 ℃ for 3 min followed by 34 cycles at 95 ℃ for 30 s, 55 ℃ for 40 s and 72 ℃ for 40 s. The mRNA expression levels of E-cadherin, vimentin, fibronectin, TGF-β1, Smad3, snail, occludin, ZO-1 and claudin were quantitatively analyzed and normalized to GAPDH levels. All assays were performed in triplicate. The forward and reverse primer sequences are provided in Table 1.

Western blot analysis

Protein extracts from rat liver tissues were quantified

Table 1 Primer sequences

E-cadherin	Forward 5′-CACACTGATGGTGAGGGTACAAGG-3′
	Reverse 5′-GGGCTTCAGGAACACATACATGG-3′
Vimentin	Forward 5′-ACCGCTTCGCCAACTACATC-3′
	Reverse 5′-GCAACTCCCTCATCTCCTCCT-3′
Fibronectin	Forward 5′-GACTCGCTTTGACTTCACCAC-3′
	Reverse 5′-ATCTCCTTCCTCGCTCAGTTC-3′
TGF-β1	Forward 5′-GAGGCGGTGCTCGCTTTGTA-3′
	Reverse 5′-GCACTGCTTCCCGAATGTCTG-3′
Smad3	Forward 5′-ATACGGATGTTCAAGTGTTCG-3′
	Reverse 5′-ACTGGGTCCTCTTTGGTTTT-3′
Snail	Forward 5′-GTCCTTGCTCCACAAACACCA-3′
	Reverse 5′-CTGCCTTCCATCAGCCATCT-3′
Occludin	Forward 5′-AGATGCTGGTTGCTGGAGAAGT-3′
	Reverse 5′-TGGAGACAGGAAACGGATGGT-3′
ZO-1	Forward 5′-CGGAGCAGAGAGGAAGAGC-3′
	Reverse 5′-GGCAGAACACCATCAGAAGG-3′
Claudin-1	Forward 5′-AGGTCTGGCGACATTAGTGG-3′
	Reverse 5′-TGGTGTTGGGTAAGAGGTTG-3′
GAPDH	Forward 5′-TGAGGACCAGGTTGTCTC-3′
	Reverse 5′-TCCACCACCCTGTTGCTGTA-3′

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

using the bicinchoninic acid (BCA) method, separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia Biotech, Piscataway, NJ, United States). Nonspecific binding was blocked with 5% nonfat milk in TBST (Tris-buffered saline with Tween) buffer for 2 h at room temperature. The membranes were incubated with primary antibody against E-cadherin (1:1000), vimentin (1:1000), fibronectin (1:1000), TGF-β1 (1:1000), Smad3 (1:1000), snail (1:1000), occludin (1:1000), ZO-1 (1:1000) or claudin (1:1000) overnight at 4 ℃ and then incubated with the horseradish peroxidase-conjugated secondary antibodies. Finally, blots were visualized using an enhanced chemiluminescence (ECL) detection kit (GE Healthcare, Amersham, United Kingdom), and GAPDH was used as a loading control. Each experiment was repeated three times independently.

Statistical analysis

All the data are presented as the mean ± SD and analyzed by one-way analysis of variance using SPSS17 software (SPSS Inc., Chicago, IL, United States). $P < 0.05$ was considered statistically significant. Histograms were generated using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA).

RESULTS

QGHXR ameliorates liver injury in rats with ALF

To evaluate the effect of QGHXR on ALF, we generated an alcohol-induced rat model of ALF and measured serum levels of ALT, AST, laminin and hyaluronidase. Rats in the untreated ALF group exhibited signs of alcohol-induced liver injury, such as fibrosis, and the ALT, AST, laminin and hyaluronidase levels in these rats were significantly higher than those in the healthy control group of rats without ALF (Figure 1). Although the QGR, HXR and QGHXR interventions reduced the levels of ALT, AST, and hyaluronidase in rats with ALF, no significant differences in laminin levels were observed (Figure 1).

QGHXR ameliorates pathological changes associated with ALF in rats

Histological images of liver pathology were also obtained. Liver sections were stained with HE or Sirius staining solution.

In the control group of rats without ALF, no detectable fatty deposits or inflammatory cell infiltrates were observed in images obtained by microscopy. In contrast, relative to the normal control group, the liver sections of rats in the untreated ALF group displayed fat droplet accumulations in hepatocytes and scattered inflammatory cell infiltrates (Figure 2A and B). In addition, collagen fibers surrounding the central vein and portal area, and overt signs of perisinusoidal fibrosis were observed in the untreated ALF group (Figure 3A and B).

Compared with the untreated ALF group, the ALF groups treated with QGR, HXR or QGHXR exhibited improvements in ALF-associated pathological changes, with the greatest improvement observed in the QGHXR-treated group (Figures 2C-E and 3C-E).

QGHXR regulates the expression of EMT-associated transcription factors

Next, we investigated whether QGHXR ameliorates liver injury in ALF rats by inhibiting TGF-β1-induced EMT. To evaluate EMT, we analyzed the expression of the epithelial marker E-cadherin, and the mesenchymal markers vimentin and fibronectin using PCR and Western blot.

PCR analysis demonstrated that E-cadherin mRNA expression decreased, and vimentin and fibronectin mRNA expression increased in rats with ALF compared with the healthy control group. However, the expression of E-cadherin in ALF rats treated with QGR, HXR or QGHXR gradually returned to the levels observed in the healthy control group (Figure 4A). In addition, a stepwise decrease in the mRNA expression of vimentin and fibronectin was observed in ALF rats treated with QGR, HXR or QGHXR. However, significant changes in the mRNA expression levels of these genes were observed only in the QGHXR-treated group (Figure 4A).

Western blot analysis revealed that E-cadherin protein levels decreased and vimentin and fibronectin protein levels increased in untreated ALF rats than those in the healthy control group, and that treatment with QGHXR, QGR or HXR reversed this effect (Figure 4B).

Collectively, these findings indicate that QGR, HXR and QGHXR inhibited the EMT in ALF rats. However,

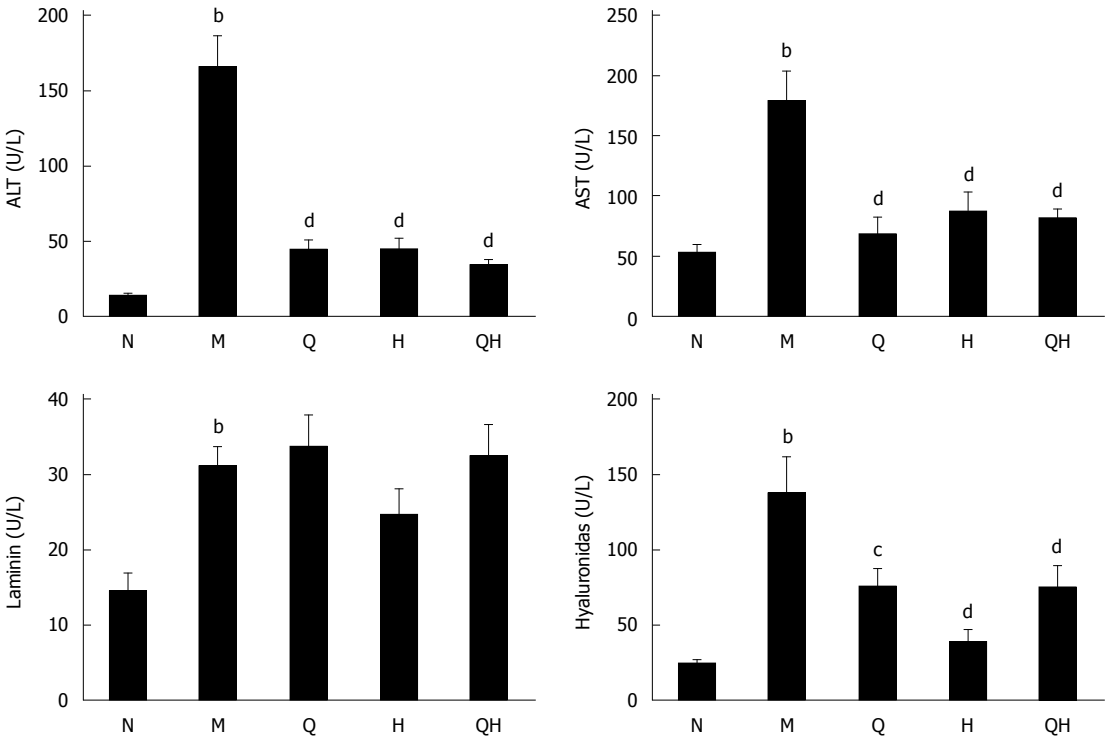
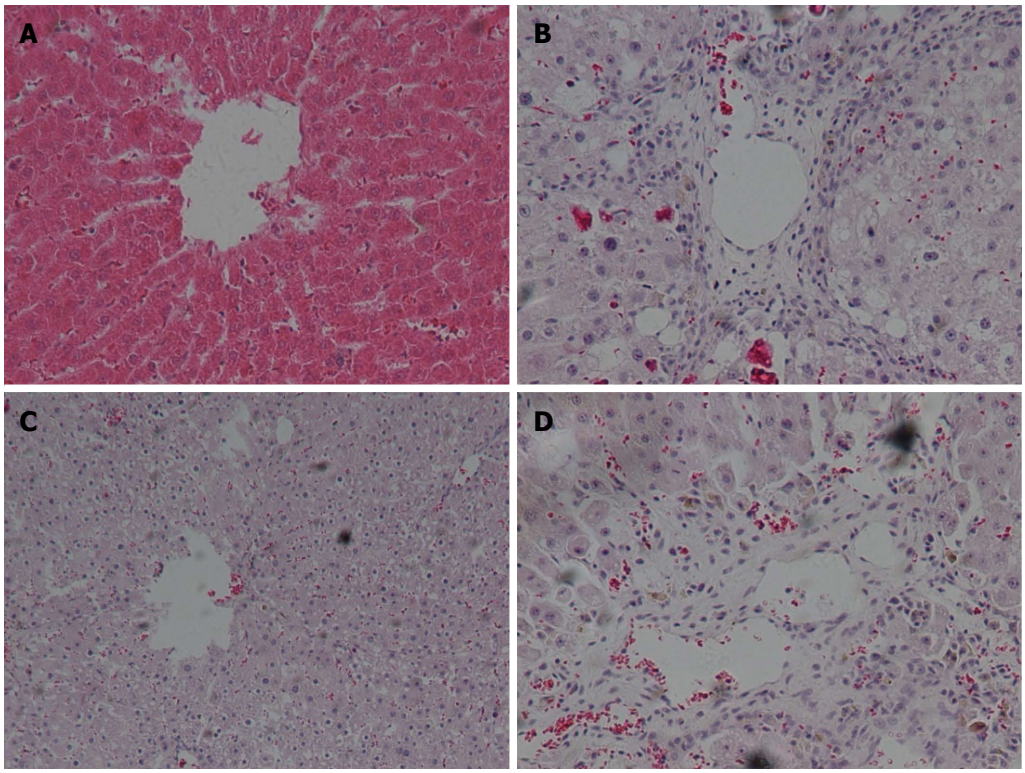


Figure 1 Qinggan Huoxue recipe ameliorates liver injury associated with alcoholic liver fibrosis with respect to alanine transaminase, aspartate transaminase, laminin and hyaluronidase levels. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* Normal healthy control group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* Model ALF group. N: Normal healthy control group; M: Model ALF group; Q: Qinggan Recipe group; H: Huoxue Recipe group; QH: Qinggan Huoxue Recipe group.



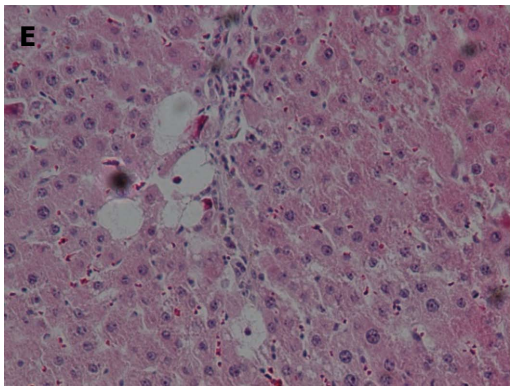


Figure 2 Changes in liver histopathology detected by hematoxylin and eosin staining and light microscopy (× 200 magnification). A: Normal healthy control group; B: Model ALF group; C: Qinggan Recipe group; D: Huoxue Recipe group; E: Qinggan Huoxue Recipe group. ALF: Alcoholic liver fibrosis.

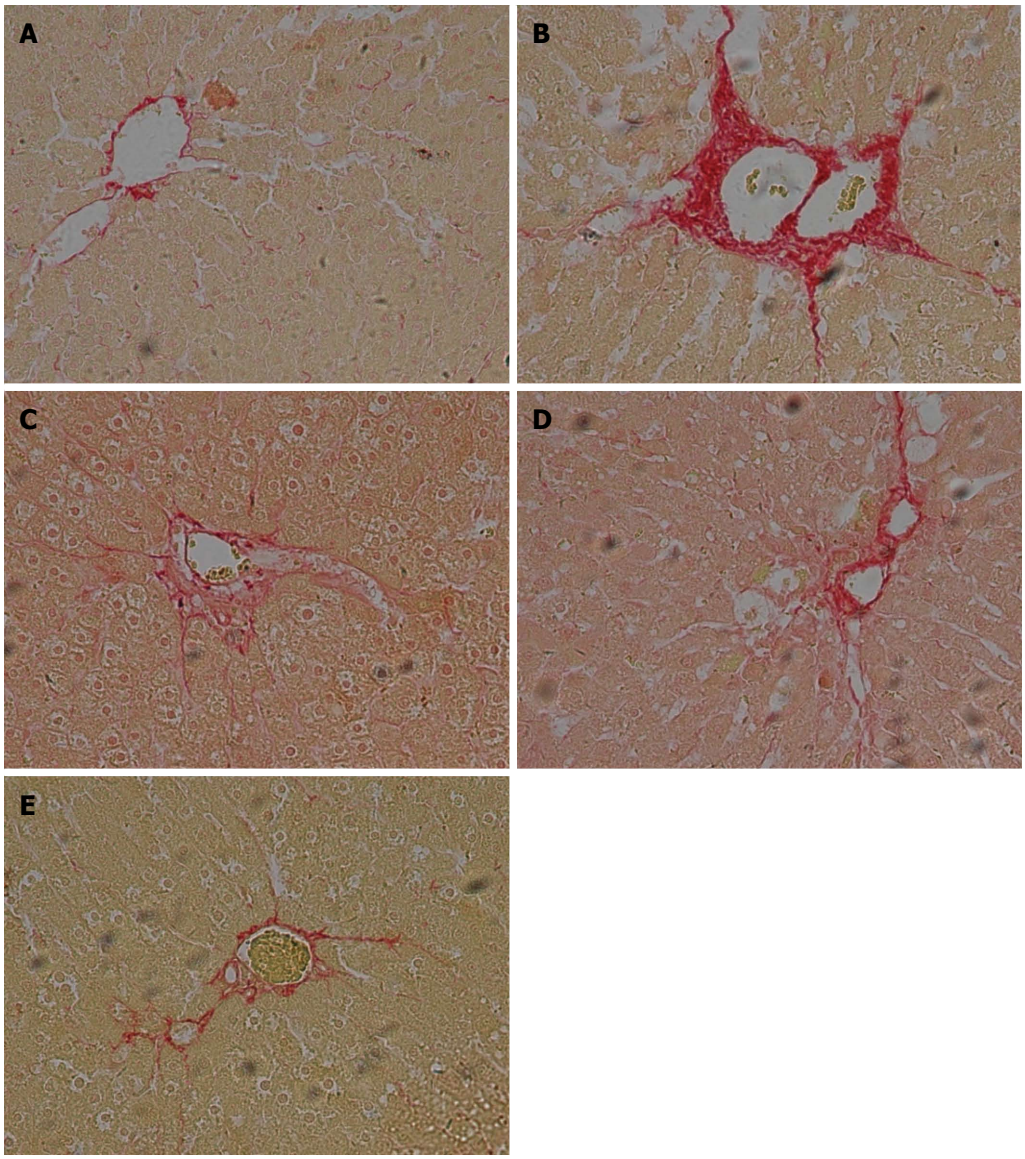


Figure 3 Changes in liver histopathology observed by Sirius Red staining and light microscopy (× 200 magnification). A: Normal healthy control group; B: Model ALF group; C: Qinggan Recipe group; D: Huoxue Recipe group; E: Qinggan Huoxue Recipe group.ALF: Alcoholic liver fibrosis.

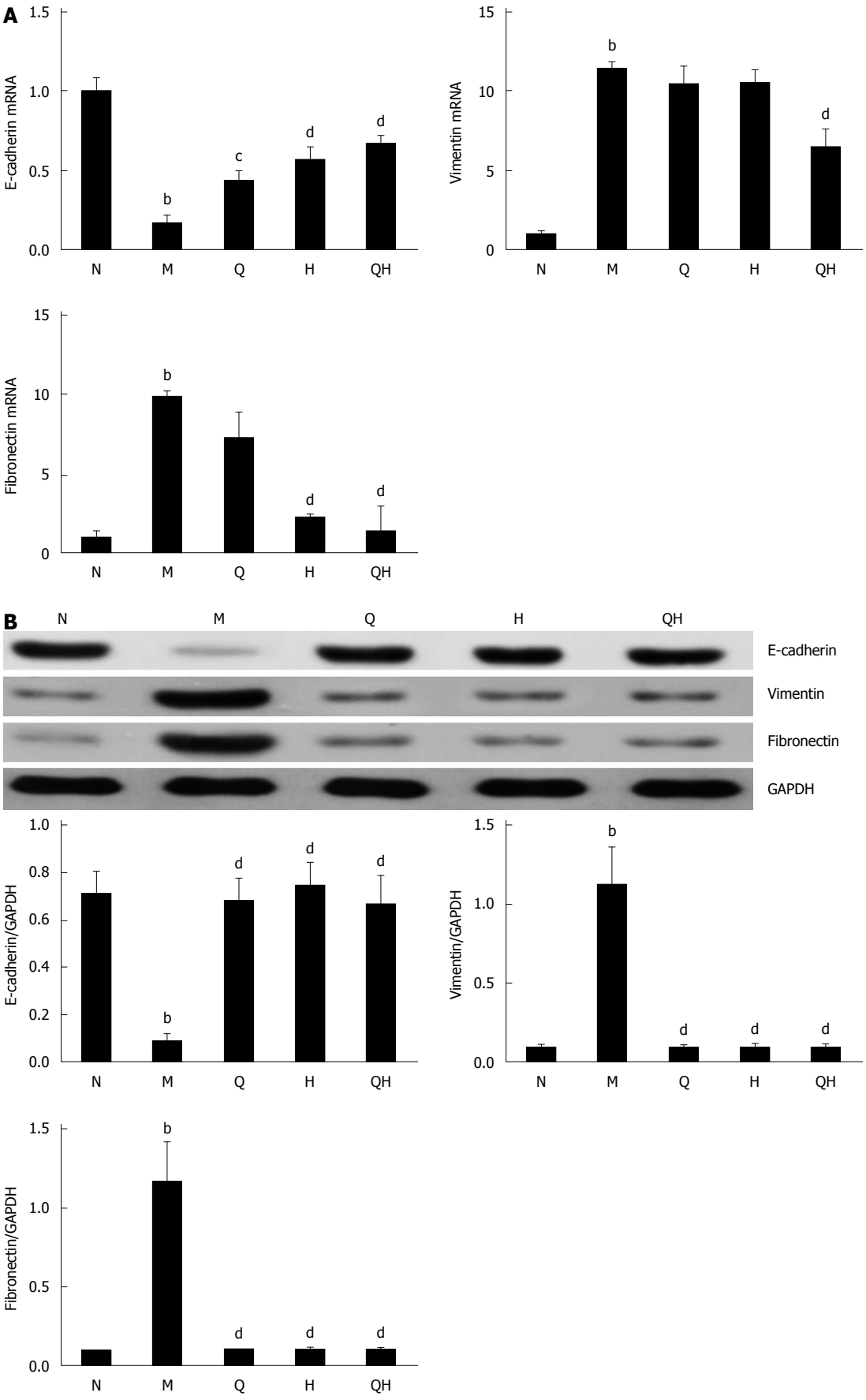


Figure 4 Qinggan Huoxue Recipe-induced expression of epithelial-to-mesenchymal transition-associated factors. A: E-cadherin, vimentin and fibronectin mRNA expression evaluated by RT-PCR; B: E-cadherin, vimentin and fibronectin protein expression evaluated by Western blot. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* Normal healthy control group; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* Model ALF group. N: Normal healthy control group; M: Model ALF group; Q: Qinggan Recipe group; H: Huoxue Recipe group; QH: Qinggan Huoxue Recipe group.

significant differences in the mRNA and protein levels of the three EMT markers were observed only in the group treated with QGHXR.

QGHXR suppresses EMT by inhibiting the TGF-β1/Smad signaling pathway

To examine the inhibitory effect of QGHXR on the expression of EMT-associated transcription factors, the expression of Snail, TGF-β1 and Smad3 was evaluated using PCR and Western blot. Compared with the healthy control group, the mRNA and protein expression of Snail was significantly up-regulated in the untreated ALF group (Figure 5); however, treatment with QGR or QGHXR inhibited the Snail mRNA and protein levels, and HXR inhibited the ALF-induced change in Snail protein levels (Figure 5). As the TGF-β/Smad signaling pathway is a critical pathway triggered by the phosphorylation of Smads, we measured the activation status of the Smad signaling pathway. As expected, TGF-β1 levels increased and Smad3 levels decreased in the liver of ALF rats. When QGR, HXR or QGHXR was given to rats with ALF, TGF-β1 and Smad3 protein levels returned to levels similar to those observed in the healthy control group (Figure 5B). The PCR results showed a similar regulation of related molecules; however, in HXR-treated rats, no significant differences in TGF-β1 mRNA levels were observed compared with the untreated control ALF group (Figure 5A). Similar trends in the mRNA and protein expression levels were observed between the three treatment groups.

In addition, the mRNA and protein expression levels of the tight junction markers occludin, ZO-1 and claudin were significantly reduced in the untreated ALF group compared with the healthy group, and treatment with QGR, HXR, or QGHXR reversed these changes (Figure 5).

DISCUSSION

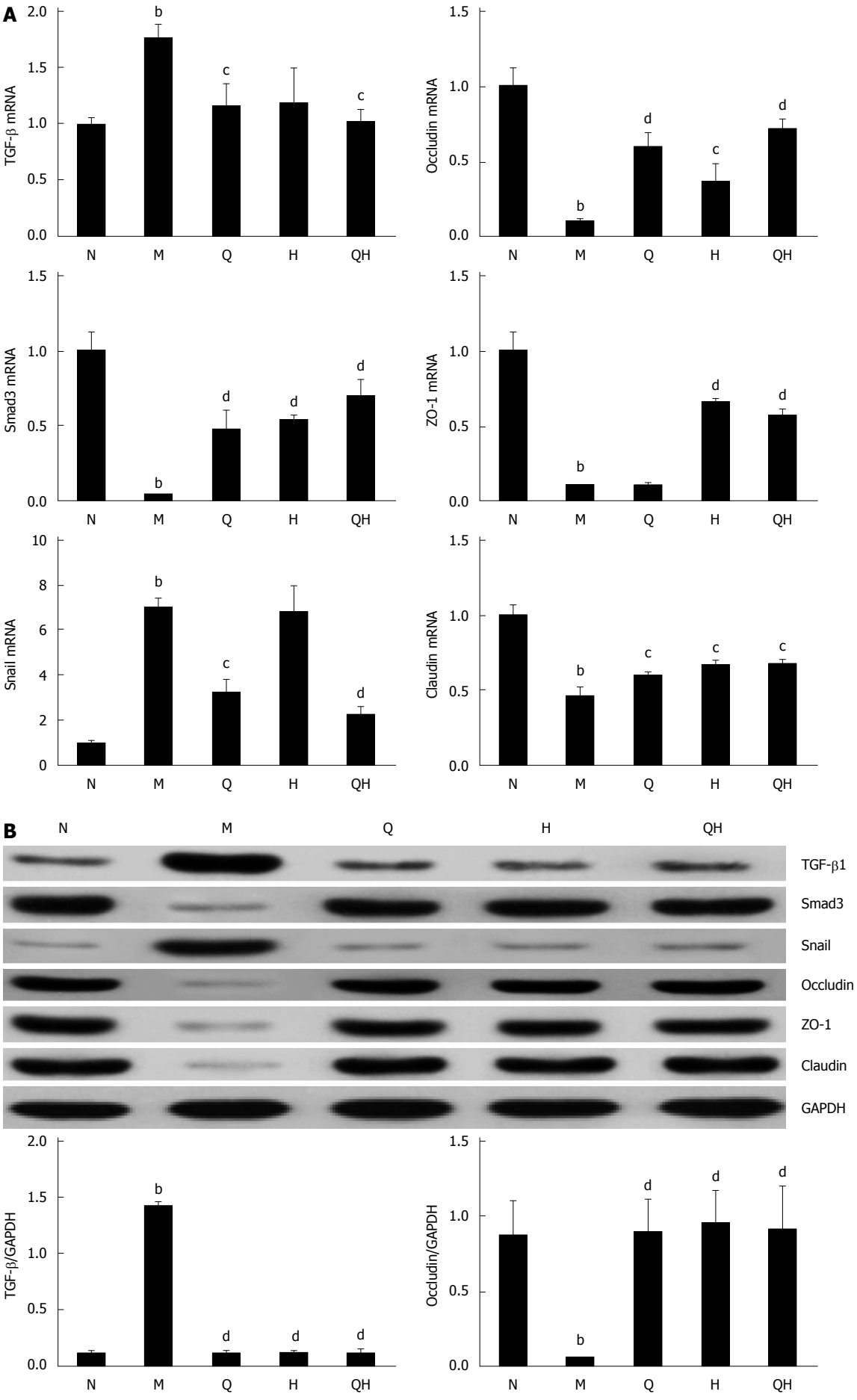
In the present study, we demonstrated that EMT was observed in rats with ALF and that QGHXR treatment can rescue the mesenchymal phenotype.

EMT is a dynamic process by which mature epithelial cells lose their defining characteristics and acquire a mesenchymal phenotype[13]. During the development of EMT, epithelial cells usually lose their adhesive capability and undergo cytoskeletal rearrangements. Previous studies have reported that EMT is characterized by loss of the expression of the epithelial marker E-cadherin and the up-regulation of the mesenchymal markers α-SMA, collagen I, fibronectin and vimentin[14,15]. In this study, E-cadherin expression decreased and vimentin and fibronectin expression increased in a rat model of ALF, indicating that an EMT model was successfully constructed.

An increasing body of evidence indicates that Chinese medicine formulas have potential value as therapeutic agents or adjuvants in ALD treatment[16]. Our previous studies revealed that QGHXR potentially exerts its therapeutic effect against liver injury *via* the LPS-KC signal transduction pathway in rats with ALD[11]. In the present study, we demonstrated that ALF rats undergo classic EMT characterized by the up-regulation of the mesenchymal markers vimentin and fibronectin, and the down-regulation of the epithelial marker E-cadherin. We observed that QGR, HXR, and QGHXR ameliorate alcoholic liver injury by reversing EMT, as demonstrated by the increase in E-cadherin expression and the decrease in vimentin and fibronectin expression (except that QGR and HXR showed no significant changes in vimentin mRNA expression, and QGR elicited no changes in fibronectin mRNA expression).

Increasing evidence indicates that TGF-β1 is the primary mediator of EMT and that the TGF-β1/Smad3 signaling pathway is important in the EMT process[17,18]. TGF-β1 binds and phosphorylates cell-surface receptors (TGF-βRI/TGF-βRII), activates TGF-βRI, and phosphorylates Smad2 or Smad3, which subsequently form a complex with Smad4[19,20]. After activation by phosphorylation and partnering with a co-Smad4, the Smad complex translocates to the nucleus and, in conjunction with other transcription factors, directs the activation and repression of genes regulated by TGF-β1[21,22]. Snail, a gene whose expression is regulated by the TGF-β/Smad signaling pathway[23], inhibited E-cadherin expression and promoted EMT. A strong inverse correlation between snail and E-cadherin expression has been reported in a panel of epithelial and dedifferentiated cells derived from carcinomas of different etiologies[24]. In the present study, the protein and mRNA expression levels of TGF-β1 increased in rats with ALF compared with normal rats. These findings are consistent with previous studies reporting that ALF is characterized by excess accumulation of collagen and other extracellular matrix proteins, steatosis, and fibrosis, and the release of the key pro-fibrogenic cytokine TGF-β1, which is mostly produced by bone marrow-derived macrophages and resident Kupffer cells[2,25,26]. The increase in snail and TGF-β1 and the decrease in Smad3 levels observed in the model ALF group further verified that the TGF-β/Smad signaling pathway is activated in ALF rats. In addition, our results demonstrated that the expression of the EMT-inducing transcription factor snail and TGF-β1 was inhibited, and the expression of Smad3 was enhanced in ALF rats treated with QGR, HXR, or QGHXR. However, the differences in snail and TGF-β1 mRNA expression induced by HXR alone were not significant (Figure 6).

EMT is characterized by the downregulation of E-cadherin expression, which results in the disruption of cell-cell junctions and the dissemination of cells from the primary tumor[27]. In hepatocytes, tight junction proteins are critical for maintaining cell polarity. It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TGF-β mRNA
Occludin mRNA
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Snail mRNA
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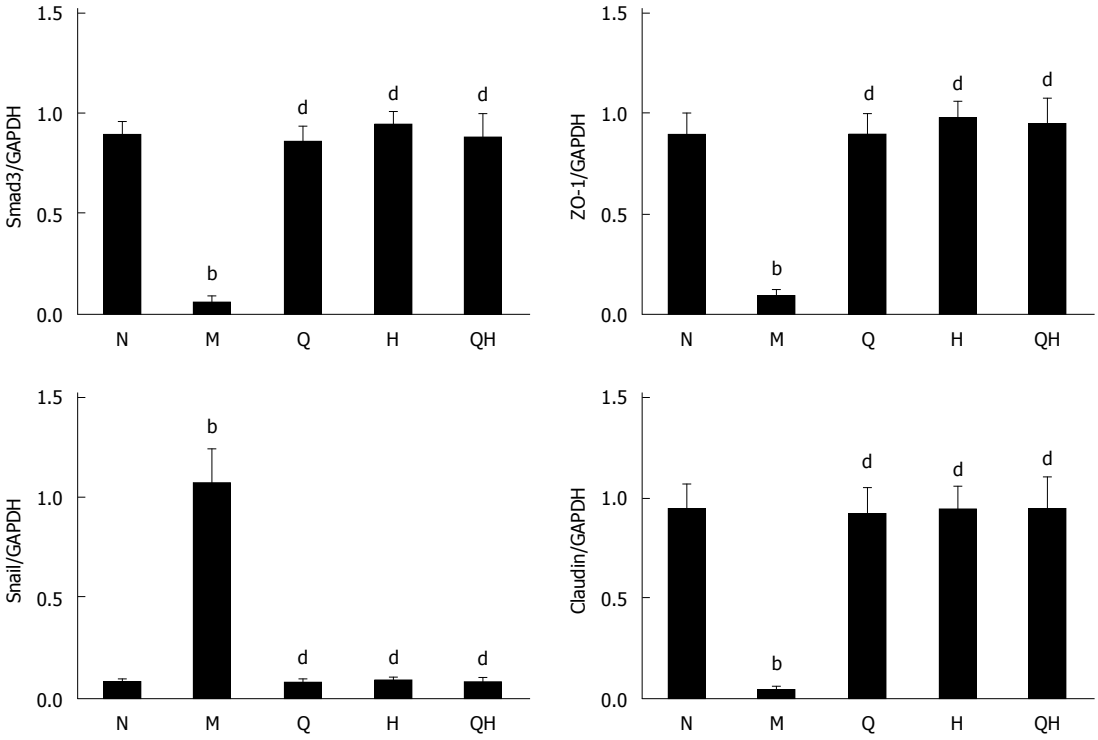

Figure 5 Qinggan Huoxue Recipe suppresses epithelial-to-mesenchymal transition by inhibiting the Smad signaling pathway. A: TGF-β1, Smad3 and Snail mRNA expression evaluated by RT-PCR; B: TGF-β1, Smad3 and Snail protein expression evaluated by Western blot. $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* Normal healthy control group; $^{c}P < 0.05$ and $^{d}P < 0.01$ *vs* Model ALF group. N: Normal healthy control group; M: Model ALF group; Q: Qinggan Recipe group; H: Huoxue Recipe group; QH: Qinggan Huoxue Recipe group.

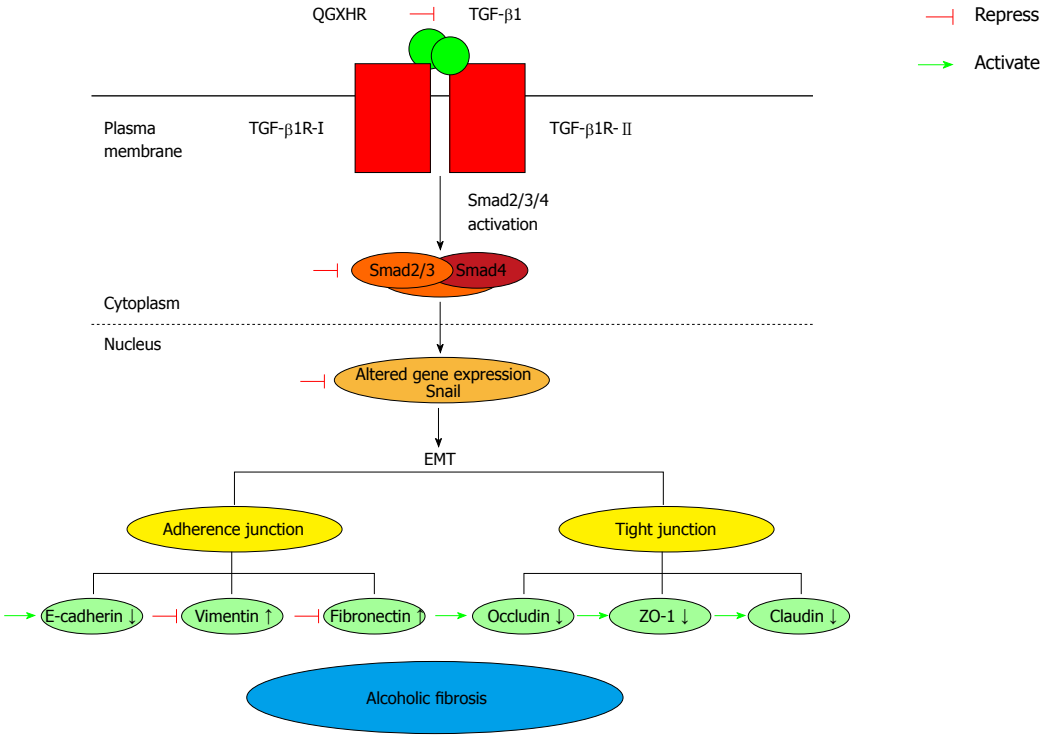

Figure 6 Potential mechanism underlying Qinggan Huoxue Recipe-mediated inhibition of epithelial-to-mesenchymal transition in alcoholic liver fibrosis rats. TGF-β1 stimulates responsive cells through binding and activating the transmembrane receptors TGF-β type I (TGF-βR-I) and type II (TGF-βR-II). Receptor ternary complexes phosphorylate and activate Smad2/3. Once activated, Smad2/3 forms heterocomplexes with Smad4, and these translocate to the nucleus and activate TGF-β1 signaling. Snail, a gene associated with the TGF-β/Smad signaling pathway, inhibits E-cadherin expression, increases vimentin and fibronectin levels, promotes EMT and decreases the levels of occludin, ZO-1 and claudin. QGR, HXR and QGHXR suppressed the effects of ALF-induced modulation of the TGF-β/Smad signaling pathway and ameliorated EMT-induced alcoholic fibrosis. QGR, HXR and QGHXR affected molecules associated with the TGF-β/Smad signaling pathway in the same manner. However, QGR and HXR exerted no significant changes in vimentin mRNA expression, QGR exerted no significant changes in fibronectin mRNA expression, and HXR exerted no significant changes in Snail and TGF-β1 mRNA expression.

is well known that claudin proteins are important components of tight junctions in paracellular transport for maintaining the structure and function[28]. Occludin and junction adhesion molecule A are transmembrane proteins that are directly involved in paracellular transport, and ZO-1 is a protein that contains a domain that forms a binding site for other tight junction proteins[29]. In the model ALF group, the mRNA and protein expression of E-cadherin, occludin and ZO-1 decreased compared with the normal healthy control group, which further verified the establishment of EMT in the ALF rat model. QGR, HXR, and QGHXR increased the mRNA and protein levels of tight junction molecules.

There are several deficiencies in our study. First, we did not evaluate the EMT markers E-cadherin, vimentin and fibronectin using immunofluorescence or immunohistochemistry. Second, we did not use gene knockout rats to verify the specific functions of the molecules analyzed in the current study. Third, although liver disease can disrupt endothelial function[30], we did not evaluate the effect of QGHXR on this parameter. Therefore, further studies are needed to verify the findings of this study.

In conclusion, our study provides evidence that QGHXR inhibits EMT in ALF by modulating the TGF-β1/Smad signaling pathway, suggesting that QGHXR ameliorates alcoholic liver injury *via* this mechanism.

COMMENTS

Background

Alcoholic liver disease has become a major cause of morbidity and mortality worldwide. EMT, a well-characterized dynamic process by which epithelial cells transform into a tissue with a fibroblastic or myofibroblastic phenotype, might have a pivotal role in inducing fibrogenesis. Therefore, targeting EMT might be a promising strategy for inhibiting the generation of extracellular matrix, which can provide a new therapeutic target for liver fibrosis.

Research frontiers

The TGF-β/Smad signaling pathway plays a pivotal role in EMT, cell proliferation and metastasis. This pathway is currently a hot topic in carcinogenesis and fibrosis studies.

Innovations and breakthroughs

EMT is a dynamic process by which mature epithelial cells lose their defining characteristics and acquire a mesenchymal phenotype. The present study demonstrated that the EMT is characterized by the down-regulation of the epithelial marker E-cadherin and the up-regulation of the mesenchymal markers vimentin and fibronectin in ALF rats. QGHXR reversed the EMT-induced changes in E-cadherin, vimentin and fibronectin levels and inhibited ALF by regulating the TGF-β1/Smad signaling pathway.

Applications

The present study provides evidence that QGHXR, a traditional Chinese medicine recipe, inhibits ALF-induced EMT by modulating the TGF-β1/Smad signaling pathway, suggesting that the potential mechanism by which QGHXR ameliorates alcoholic-induced liver injury is associated with this pathway. Thus, EMT represents a novel therapeutic target for ALF and the findings of this study provide new insight into the pathogenesis of ALF.

Terminology

EMT is a phenotypic conversion of the epithelium to a fibroblastic or myofibroblastic phenotype. EMT begins with the dissociation of adhesions between epithelial cells, the decrease of apical-basal polarity, the reorganization of the actin cytoskeleton and the increase of cell motility.

Peer-review

This study "Qinggan Huoxue Recipe suppresses epithelial-to-mesenchymal transition in alcoholic liver fibrosis through TGF-β1/Smad signaling pathway" is very interesting.

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ORIGINAL ARTICLE

Basic Study

Laparoscopic colonic anastomosis using a degradable stent in a porcine model

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Author contributions: Ma L performed the majority of experiments and analyzed the data; Ma L, Wang HH, Huang DY, Ge GJ, Hu HY and Yu SC contributed equally to treatment of animals; Cai XJ and Ma L designed and coordinated the research; Ma L and Yu YL wrote the paper.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of Sir Run Run Shaw Hospital, Hangzhou, China.

Institutional animal care and use committee statement: All procedures were carried out according to the Institutional Animal Care and Use Committee Guide of Center for Drug Safety Evaluation and Research of Zhejiang University with the following reference number: IACUC-13001.

Conflict-of-interest statement: None of the authors disclosed any conflict of interest.

Data sharing statement: No additional data are available.

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Abstract

AIM: To explore the feasibility and safety of laparoscopic colonic anastomosis using a degradable stent in a porcine model.

METHODS: Twenty Bama mini-pigs were randomly assigned to a stent group (n = 10) and control group (hand-sewn anastomosis, n = 10). The anastomotic completion and operation times were recorded, along with histological examination, postoperative general condition, complications, mortality, bursting pressure, and the average anastomotic circumference (AC).

RESULTS: All pigs survived postoperatively except for one in the stent group that died from ileus at 11 wk postoperatively. The operation and anastomotic completion times of the stent group were significantly shorter than those of the control group (P = 0.004 and P = 0.001, respectively). There were no significant differences in bursting pressure between the groups (P = 0.751). No obvious difference was found between the AC and normal circumference in the stent group, but AC was significantly less than normal circumference

in the control group (P = 0.047, P < 0.05). No intestinal leakage and luminal stenosis occurred in the stent group. Histological examination revealed that the stent group presented with lower general inflammation and better healing.

CONCLUSION: Laparoscopic colonic anastomosis with a degradable stent is a simple, rapid, and safe procedure in this porcine model.

Key words: Laparoscope; Colon; Anastomosis; Stent; Porcine model

Core tip: We explored the feasibility and safety of laparoscopic colonic anastomosis using a degradable stent in a porcine model. Twenty Bama mini-pigs were randomly assigned to a stent group and hand-sewn anastomosis group. The operation and anastomotic completion times of the stent group were significantly shorter than those of the control group. There was no significant difference between the anastomotic and normal circumference in the stent group. No intestinal leakage and luminal stenosis occurred in the stent group. Histological examination of anastomoses revealed that the stent group presented with less general inflammation and better healing than the control group.

Ma L, Cai XJ, Wang HH, Yu YL, Huang DY, Ge GJ, Hu HY, Yu SC. Laparoscopic colonic anastomosis using a degradable stent in a porcine model. *World J Gastroenterol* 2016; 22(19): 4707-4715 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i19/4707.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i19.4707

INTRODUCTION

There is substantial evidence that laparoscopic surgery plays a pivotal role in abdominal surgical disease[1,2]. Laparoscopic intestinal anastomosis is a basic technique in gastrointestinal procedures. The hand-sewn method has been the most widely accepted procedure for intestinal anastomosis for the past 160 years. Although surgical skills have been significantly improved and developed, hand-sewn anastomoses are still complicated and time-consuming[3]. An effective alternative technique is stapled anastomosis. Although this is relatively convenient and time-saving, stapler devices are more expensive than the hand-sewn method and have a higher risk of anastomotic stenosis[4,5]. Besides, the foreign materials in anastomosis may induce chronic inflammation[6,7], which could slow down the process of healing or lead to secondary leakage and stenosis[8,9]. In addition, intestinal staplers cannot be used for hemostasis, and suturing after stapling may be required[10]. Another feasible technique is sutureless anastomosis, such as compression rings, tissue glue, and laser anastomosis, which have been in use since Murphy's button in 1892[10-13]. However, sutureless anastomosis is less applied clinically due to some safety issues, such as weak anastomotic strength, necrosis, and stricture. Therefore, an ideal laparoscopic surgical procedure that can achieve the desired results is urgently needed.

In our previous experiments, we have exhibited the feasibility and safety of colonic anastomosis and primary repair of colonic perforation using a degradable stent in a porcine model undergoing open surgery[14-16]. In this study, we further explored the application of this stent in laparoscopic colonic anastomosis using a degradable stent.

Figure 1 Appearance of degradable stent.

MATERIALS AND METHODS

Animals

Twenty healthy experimental Bama mini-pigs of either sex, weighing approximately 30 kg, were purchased from Shanghai Multi-Bio-Sci-Tech Co. Ltd., China. Animal were raised separately in clean cages at the Experimental Animal Center of Zhejiang University and provided with a liquid diet for 5 d before surgery. They were then were starved for 12 h and fed with 5% magnesium sulfate to clean the colonic lumen. Cefazolin sodium was administered intramuscularly for preoperative antibiotic prophylaxis. All the protocols were approved by the Experimental Animal Ethics Committee of Zhejiang University.

Features of the degradable stent

The stent was developed by the Institute of Polymer Science of Zhejiang University (Figure 1). The material properties were demonstrated in our previous studies[14-16]. The stent is synthesized from 1,3-propanediol, 1,2-propanediol, and sebacic acid and decomposes finally to CO_2 and water. *In vivo*, we found that the stents were damage free on day 10 and were degraded and broken on day 28. The stent was approved by the State Food and Drug Administration of

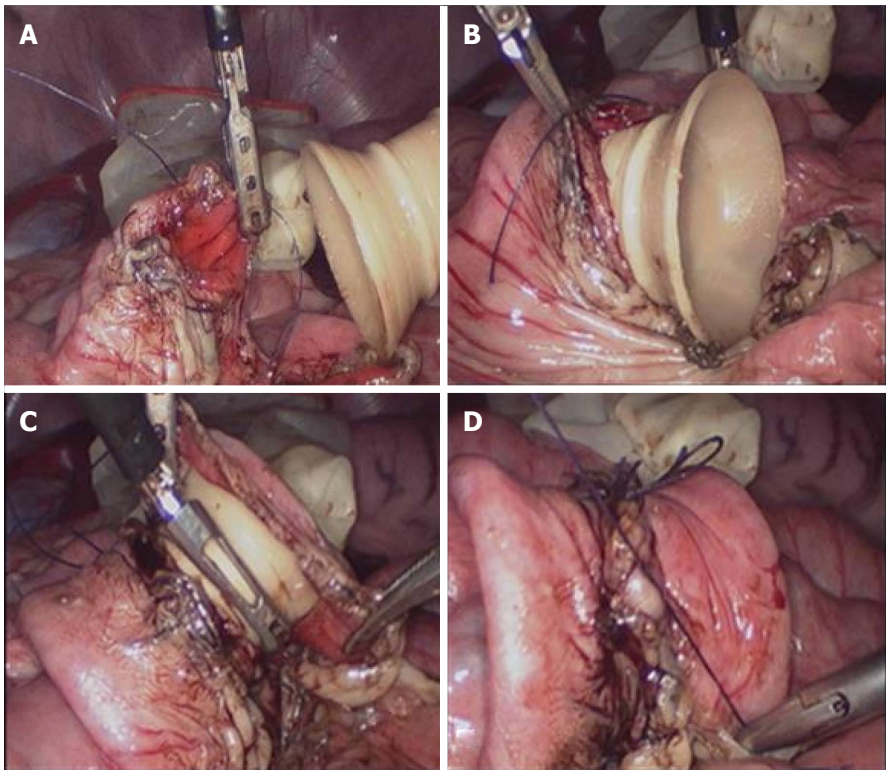

Figure 2 Operative procedure in the stent group. A: A purse-string suture was performed circularly around the intestine; B: One end of the stent was embedded in one side of the intestinal cavity, and the purse string was tightened, knotted, and fixed; C: The other side of the stent was placed in the contralateral intestinal cavity, and the purse string was tightened, knotted, and fixed; D: Both sides of the purse-string were knotted and fixed.

China for its biocompatible qualities (No. G20090993)[16].

Experimental design and procedure

Twenty pigs were randomized and assigned to a degradable stent group (n = 10) and control group (hand-sewn anastomosis, n = 10). The pigs in each group were divided evenly into two subgroups according to the time of sacrifice (2 wk and 3 mo postoperatively), and each subgroup included five pigs. A laparoscopic colonic anastomosis with a degradable stent was performed in the stent group, and laparoscopic hand-sewn anastomosis was performed in the control group.

The animals were anesthetized by intramuscular injection of midazolam (total 5 mg, Jiangsu Enhua Pharmaceutical Group, China) and inhalation anesthesia with isoflurane, with subsequent intubation and mechanical ventilation. The animals were in the supine position on a disposable disinfectant towel. Laparotomy was performed *via* left lower quadrant incision (about 2.5 cm), and pneumoperitoneum was established using CO_2 to create a sufficient operating space in the abdominal cavity. The appropriate intestinal segment was selected, and then the colon and partial mesentery was dissociated. The tissue with bad blood supply was cut, and the colonic contents were removed. The colonic lumen was cleaned, and the incisal edge was disinfected. At 0.5 cm from both ends of the bowel transection, a purse-string suture using 3-0 absorbable thread sutures was performed circularly around the intestine at the seromuscular layer of the colon (Figure 2A). One end of the stent was embedded into the intestinal cavity, and the purse string was tightened, knotted, and fixed (Figure 2B). Afterwards, the other end of the stent was placed into the contralateral intestinal cavity, and the purse string was tightened, knotted, and fixed (Figure 2C). Finally, both sides of the purse-string were knotted and fixed, and another one or two stitches were added intermittently, if necessary, to avoid intestinal volvulus (Figure 2D). No abdominal drain was placed, and the incisions were closed. Pigs in the control group received a hand-sewn, end-to-end colonic anastomosis. The procedure was performed as previously described[15].

After the operation, pigs were given free access to water for 24 h and returned to a fluid diet 24 h later, with normal diet being resumed on postoperative day 7. The anastomotic completion and operation times of each group were recorded as well as postoperative general condition, complications, and mortality. Five pigs in each group were sacrificed 2 wk postoperatively to evaluate the bursting pressure, and the rest was sacrificed 3 mo postoperatively to assess the average anastomotic circumference (AC) and healing of the anastomosis. All the procedures were conducted by the same operators. The circumference was approximately 10 cm above and below the anastomotic stoma, and the narrowest circumference was measured. After

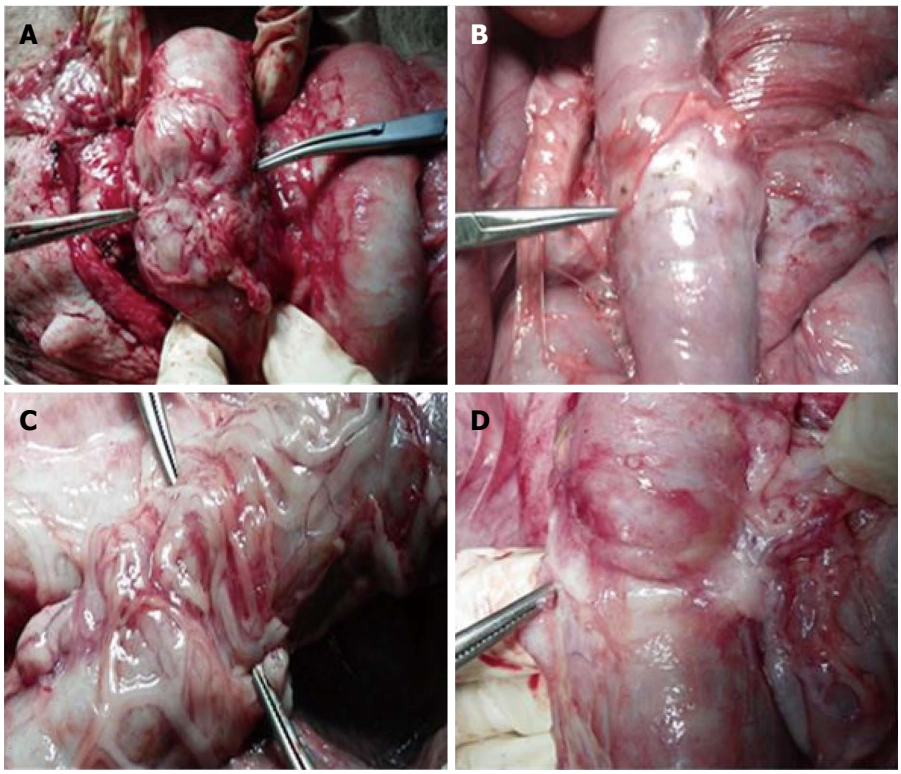

Figure 3 General observation of the anastomosis. A: Stent group at postoperative week 2; B: Stent group at postoperative month 3; C: Control group at postoperative week 2; D: Control group at postoperative month 3.

sacrifice, an approximately 5-cm anastomotic segment was resected for histological examination, including hematoxylin-eosin (HE), Masson's trichrome stain, and immunohistochemical staining, performed by the Pathology Department of Sir Run Run Shaw Hospital, Hangzhou, China. The samples were fixed, embedded, and sliced into 5-μm thick sections. Immunohistochemical staining was performed with mouse anti-porcine anti-α-smooth muscle actin (SMA) antibody (1:300; Abcam, Cambridge, United Kingdom), anti-basic fibroblast growth factor (b-FGF) antibody (1:300, Santa Cruz Biotechnology, Dallas, TX, United States), or anti-transforming growth factor (TGF)-β1 antibody (1:300, Santa Cruz Biotechnology). The second antibody was performed using rabbit anti-mouse (1:1000, Zhongshan Goldenbridge Biotechnology Co. Ltd., Beijing, China).

Statistical analysis

Continuous variables, presented as mean ± SD, were compared using Student's *t* test. Statistical analysis was performed by using SPSS version 18.0 (SPSS Inc., Chicago, IL, United States).

Animal care and use statement

All procedures were carried out according to the Institutional Animal Care and Use Committee Guide of Center for Drug Safety Evaluation and Research of Zhejiang University with the reference number: IACUC-13001.

RESULTS

General condition

General observation of anastomosis is shown in Figure 3. One pig in the stent group died from ileus at postoperative week 11. Autopsy found that the ileus was located below the anastomosis, and the cause of death was congenital intestinal valvular disease. The other animals all survived.

Anastomotic completion and operation times

As shown in Table 1, the mean operation time of the stent group was significantly shorter than that of the control group ($P = 0.004$, $P < 0.01$). Within the stent group, there were significant differences between the two subgroups ($P = 0.010$). The operation time in the 2 wk and 3 mo subgroups was 86.6 ± 10.9 min and 56.6 ± 16.6, respectively. However, no significant differences were found in the two subgroups of the control group ($P = 0.426$). The mean anastomotic completion time of the stent and control groups was 33.1 ± 18.5 min and 65.5 ± 19.9 min, respectively. There was a significant difference between the two groups ($P = 0.001$). In the stent group, the anastomotic completion time of the 2 wk subgroup was less than that of the 3 mo subgroup ($P = 0.016$). In addition, there were no significant differences in the

Table 1 Parameters compared between different groups

Groups	Subgroups	Operation time(min)	Completion time (min)	Bursting pressure (cmH_2O)	Anastomotic circumference (cm)	Normal circumference (cm)
Stent group		71.6 ± 20.6	33.1 ± 18.5	-	-	-
	Week 2	56.6 ± 16.6	23.6 ± 14.8	108.0 ± 34.9		-
	Month 3	86.6 ± 10.9	42.6 ± 18.0	-	7.7 ± 0.4	7.7 ± 0.2
Control group		108.3 ± 27.8	65.5 ± 19.9	-	-	-
	Week 2	100.8 ± 34.1	57.2 ± 24.3	97.5 ± 60.2	-	-
	Month 3	115.8 ± 20.8	73.8 ± 11.5	-	7.1 ± 1.1	8.3 ± 1.1

two subgroups of the control group (P = 0.204).

Bursting pressure and AC

The bursting pressure of the 2 wk subgroup in the stent and control groups was 108.0 ± 34.9 and 97.5 ± 60.2 cm H_2O, respectively, and there were no significant differences in the two subgroups (P = 0.751). The normal intestinal circumference and AC of the 3 mo subgroups in the stent and the control groups were 7.7 ± 0.2, 7.7 ± 0.4, 8.3 ± 1.1, and 7.1 ± 1.1 cm, respectively. There were no significant differences between the normal intestinal circumference and the AC in the stent group (P = 0.344). However, the AC was lower than the normal intestinal circumference in the control group (P = 0.047).

Colonic anastomotic healing and stent degradation

No intestinal leakage or luminal stenosis occurred in the stent group; while leakage appeared in three pigs in the 2 wk subgroup of the control group, and two pigs achieved healing. Bowel tapering was found in two pigs in the 3 mo subgroup of the control group. No stents were dislocated in the 2 wk subgroups of both groups, but the stents presented with partial breakages because the material properties were fragile. All the stents were absorbed in anastomotic stoma at 3 mo postoperatively.

Microscopic findings at 2 wk and 3 mo postoperatively

HE staining at postoperative week 2 in both groups showed that the anastomotic mucosa was absent, and granulation tissue formed with infiltration of a large number of lymphocytes, plasma cells, and neutrophils. Fibrous tissue hyperplasia, collagen deposition, and muscularis propria interruption were present. However, the degree of inflammatory infiltration in the stent group was lower than in the control group (Figure 4A-D). Collagen deposition was similar in the two groups (Figure 5A-D). Immunohistochemical staining demonstrated that α-SMA had higher immunostaining intensity and wider range than b-FGF and TGF-β1 and no significant differences were found between the two groups (Figure 6A and B).

HE staining at postoperative month 3 showed that colonic mucosa was present at the site of anastomosis, along with fibrous tissue hyperplasia and collagen deposition that extended through the adventitial layer. Smooth muscle bundles were also interleaved between the mucosa and adventitial layer, fusing with the muscularis mucosa. However, submucosa was absent at the site of the anastomosis. The amount of smooth muscle tissue in the stent group was higher than in the control group (Figure 4E-H). There was a large amount of collagen deposition and scar tissue formation and no obvious differences were found between the two groups (Figure 5E-H). Immunohistochemical staining showed that the intensity of α-SMA staining decreased compared with that at postoperative week 2, but the range was still extensive. Positive staining smooth muscle cells presented with brown color and were arranged in a fascicular pattern. The immunostaining intensity and range of b-FGF and TGF-β1 were lower than those at week 2 (Figure 6).

DISCUSSION

In the present study, the safety and feasibility of laparoscopic colonic anastomosis using a degradable stent was investigated by comparing with hand-sewn anastomosis. The operation and anastomotic completion times using a degradable stent were significantly shorter than those for the hand-sewn method, while no significant differences were found in bursting pressure. Besides, no obvious significance was found between the AC and normal circumference in the stent group, whereas the AC was significantly less than the normal circumference in the control group. Severe complications, such as intestinal leakage and luminal stenosis, were also less frequent than those with the hand-sewn method. Therefore, we conclude that the simple surgical procedure with a degradable stent was a simple, rapid, feasible, and safe procedure in this porcine model.

The methods for intestinal anastomosis are numerous, and each has its advantages and disadvantages. Extracorporeal anastomosis is simple but is highly invasive. Therefore, totally laparoscopic procedures have been gradually paid more attention. Laparoscopic stapled anastomosis may elicit local inflammation caused by a foreign body reaction and result in leakage and stenosis[17]. The biodegradable compression ring (BAR Valtrac) is hard to perform and is not suitable for laparoscopic operation. Another novel compression anastomosis clip (Hand CAC 30), which is made of a shape-memory alloy of nickel-titanium, is suitable for laparoscopic operation[18-20].

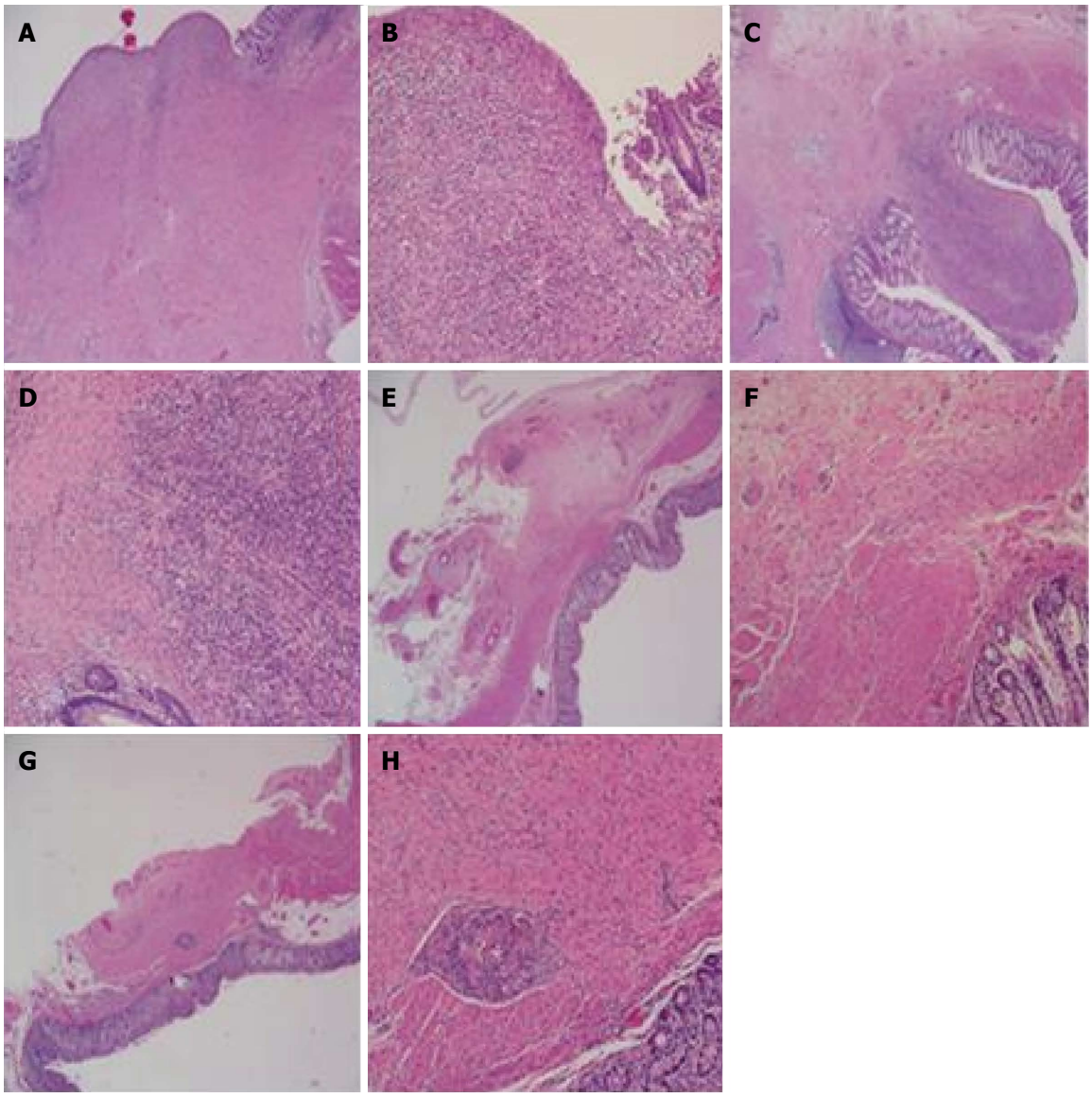

Figure 4 Hematoxylin and eosin staining of anastomosis. A, B: Hematoxylin and eosin (HE) staining of the stent group at 2 wk postoperatively (× 100, × 200); C, D: HE staining of the control group at 2 wk postoperatively (× 100, × 200); E, F: HE staining of the stent group at 3 mo postoperatively (× 100, × 200); G, H: HE staining of the control group at 3 mo postoperatively (× 100, × 200).

However, CAC is metallic, which cannot be degraded and removal and discharge are difficult[21]. Therefore, it is essential to develop an ideal surgical procedure that is easy to perform. In the present study, we introduced a novel degradable stent that was synthesized from 1,3-propanediol, 1,2-propanediol and sebacic acid, which can decompose finally to CO_2 and water. Like the biodegradable anastomosis, the degradable stent leaves no residual foreign body in the anastomosis and can isolate the intestinal contents from the anastomotic stoma, subsequently reducing the chance of infection. In addition, the appropriate stent diameter can be selected, which may avoid anastomotic stenosis and achieve the ideal effect.

Twenty healthy experimental Bama mini-pigs were used in our study. Only one pig died from ileus caused by congenital intestinal valvular disease. Hence, the death was not directly related to the experimental method. The operation and anastomotic completion times in the stent group were significantly shorter than in the control group, indicating that the stent procedure was easier and simpler to perform compared with the hand-sewn method. In the stent group, the operation time in the 3 mo subgroup declined 34.65% more than in the 2 wk subgroup, while the anastomotic completion time declined by 44.6% in both subgroups. The anastomotic completion time for the last four pigs in the stent group was 18, 15, 18, and 17 min, respectively. This was superior to the hand-sewn method (24.5 ± 11.3 min) and close to the stapled intestinal anastomosis (mean 15.5 ± 7.8 min)[22]. The results indicated the potential of the laparoscopic colonic anastomosis stent method. No significant differences were found in bursting pressure between the groups. Bursting pressure is one of the most reliable parameters for measuring the quality of the healing process. The

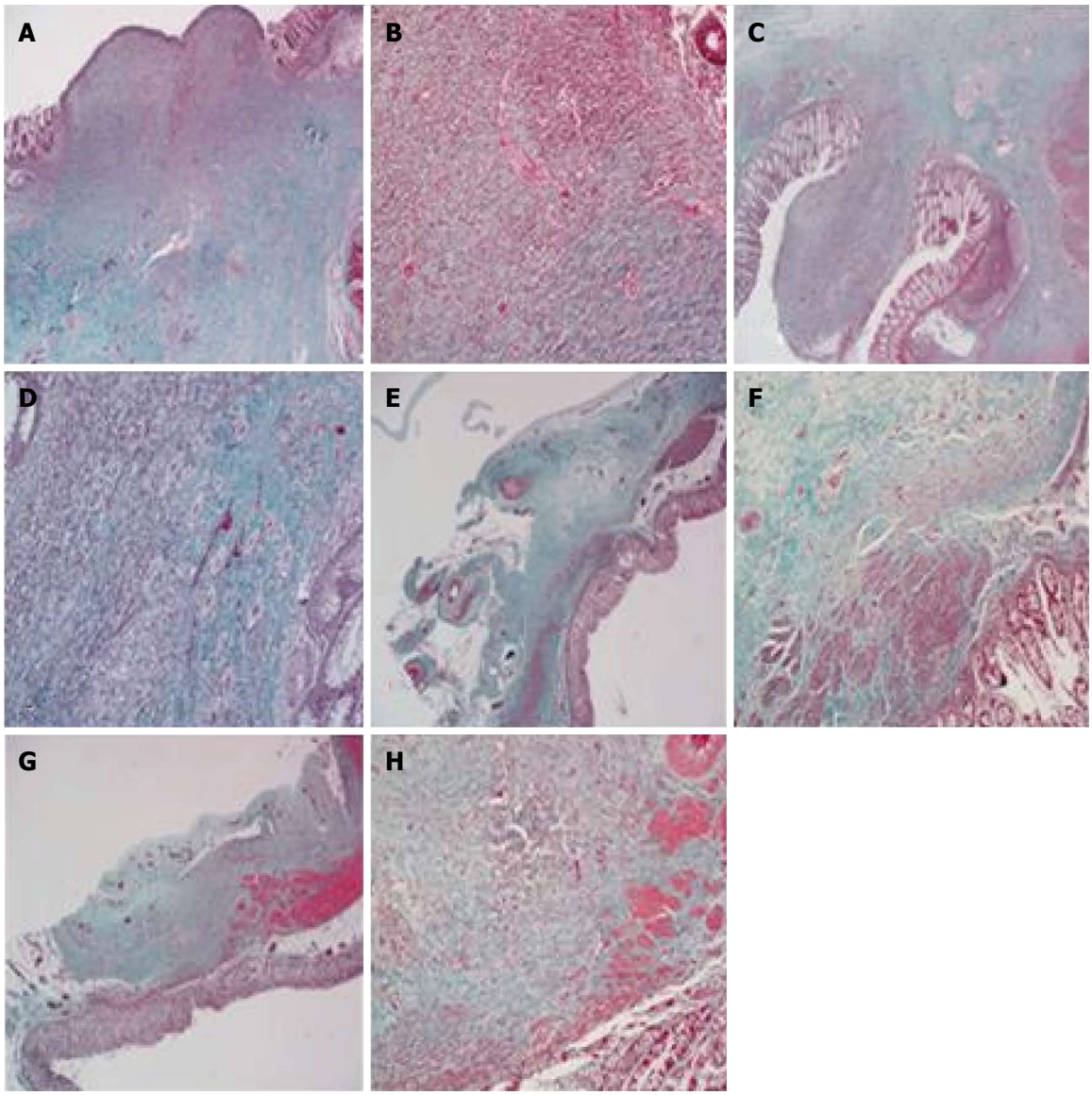

Figure 5 Masson's trichrome staining of anastomosis. A, B: Masson's trichrome staining of the stent group at 2 wk postoperatively (× 100, × 200); C, D: Masson's trichrome staining of the control group at 2 wk postoperatively (× 100, × 200); E, F: Masson's trichrome staining of the stent group at 3 mo postoperatively (× 100, × 200); G, H: Masson's trichrome staining of the control group at 3 mo postoperatively (× 100, × 200). The hyperplastic fibrous tissue and collagenous fibers are shown with green or blue, while hyperplastic smooth muscle cells with red.

healing process in the stent and control groups was uneventful. There were no significant differences between the average AC and normal circumference in the stent group at 3 mo postoperatively, which showed that no luminal stenosis occurred in the presence of the stents. However, the average AC of the control group at 3 mo postoperatively was less than the normal average circumference, which may be the reason for bowel tapering in two pigs.

Histological examination is the gold standard for confirming anastomotic healing. We found that the mucosa of the anastomosis was absent at postoperative week 2, and granulation tissue formed with a large amount of inflammatory cell infiltration. In addition, fibrous tissue hyperplasia and collagen deposition were noted, but the smooth muscle layer was not formed. The degree of inflammatory infiltration in the stent group was lower than in the control group. The mucosa had grown completely, and smooth muscle was already formed at 3 mo postoperatively. The amount of smooth muscle in the stent group was more than in the control group, and foreign body granuloma was noted in the control group. The results indicated that the healing was similar in the two groups, but the inflammatory reaction was lighter and the growth of intestinal smooth muscle was better in the stent group than in the control group. Therefore, the stent method had some advantages. No foreign body, lower tension, and isolation from intestinal contents might have contributed to the satisfactory results in the stent group.

However, there were some limitations in our study. The sample of animals was small, and the experimental animals were Bama mini-pigs. Although

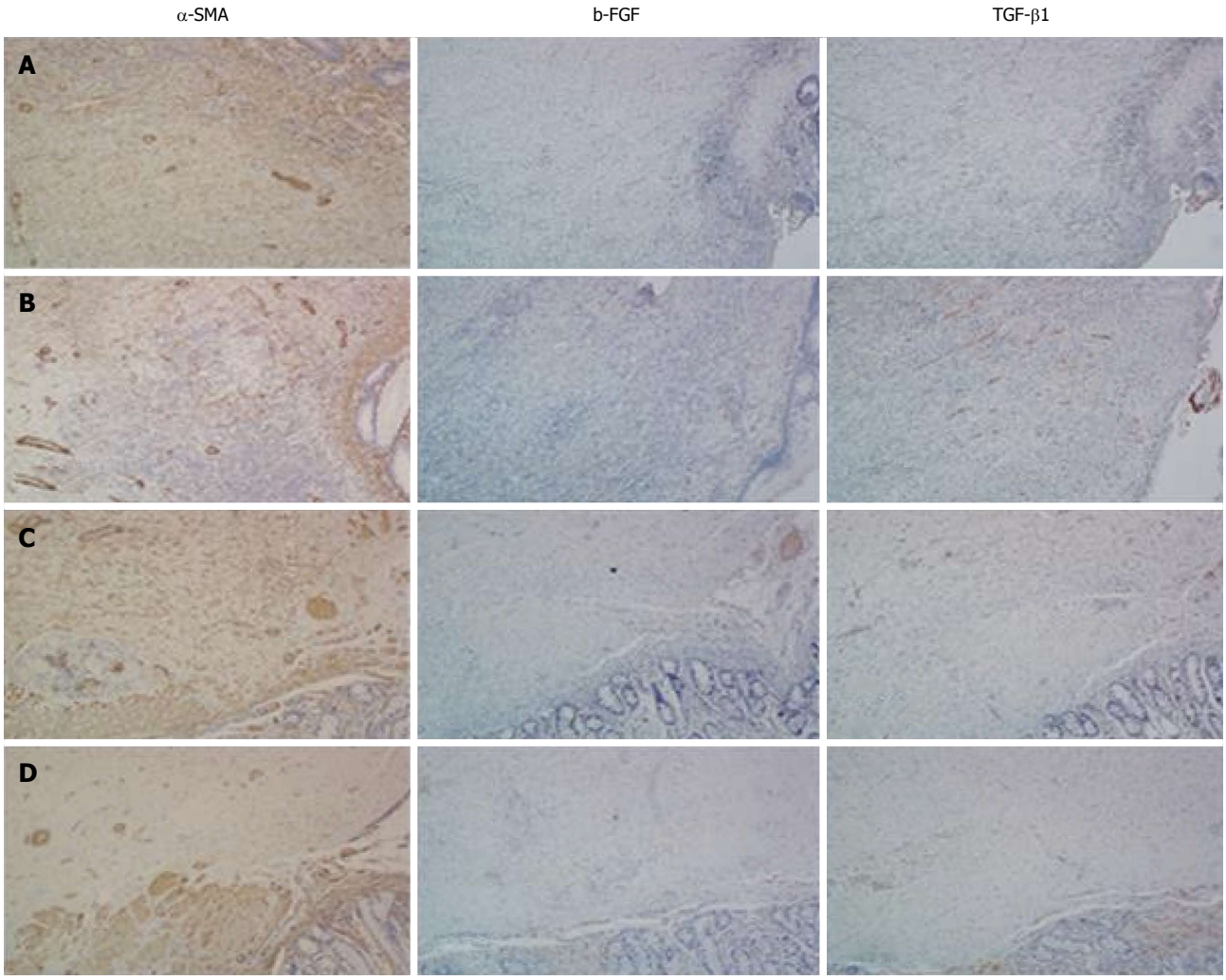

Figure 6 Immunohistochemical staining of anastomosis. A: Immunohistochemical staining for α-SMA, b-FGF, and TGF-β1 of the stent group at 2 wk postoperatively; B: Immunohistochemical staining for α-SMA, b-FGF and TGF-β1 of the control group at 2 wk postoperatively; C: Immunohistochemical staining for α-SMA, b-FGF and TGF-β1 of the stent group at 3 mo postoperatively; D: Immunohistochemical staining for α-SMA, b-FGF and TGF-β1 in the control group at 3 mo postoperatively (all × 200). α-SMA: α-smooth muscle actin; b-FGF: Basic fibroblast growth factor; TGF: Transforming growth factor.

the animals have similar physiological features to humans and comparable operating procedures, the healing ability and anti-infective activity in the animals might not be consistent with those of humans. Hence, further research should be done before the procedure is applied clinically.

In conclusion, laparoscopic colonic anastomosis with degradable stents could be a potential alternative procedure for intestinal anastomosis.

COMMENTS

Background

Laparoscopic intestinal anastomosis is a basic technique in gastrointestinal procedures. Although surgical skills have been significantly improved and developed, hand-sewn anastomoses are still complicated and time-consuming. Therefore, an ideal laparoscopic surgical procedure that can achieve the desired results is urgently needed.

Research frontiers

Many procedures have been developed, including stapling devices, compression rings, tissue glue, and laser anastomosis. However, stapler devices are expensive and require introduction of a permanent foreign body. Sutureless anastomosis has weak anastomotic strength, necrosis, and stricture. Tissue glue and laser anastomosis are not used clinically because of weak anastomotic strength.

Innovations and breakthroughs

The authors developed a degradable stent and have exhibited its feasibility and safety in colonic anastomosis and primary repair of colonic perforation in a porcine model undergoing open surgery. With this simple technique, suturing time was greatly decreased, the anastomosis was free of compressive pressure, and the damage to the submucosal vascular plexus and mesenteric vessels were minimized. In this study, the application of this stent in laparoscopic colonic anastomosis was explored.

Applications

Laparoscopic colonic anastomosis with degradable stents could be a potential alternative procedure for intestinal anastomosis. Further research should be done before the procedure is applied clinically.

Terminology

Anastomotic circumference: The circumference of the anastomosis. Bursting pressure: the maximum pressure the segment resisted or the pressure at the

moment the first leakage.

Peer-review

This is a nice and novel study with good practical value. The novel technique described has potential applications in the future.

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ORIGINAL ARTICLE

Basic Study

Plasma long noncoding RNA expression profile identified by microarray in patients with Crohn's disease

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Author contributions: Chen D and Liu J performed the majority of experiments; Zhao HY, Chen YP and Xiang Z participated in clinical sample collection, microarray, qRT-PCR and data analysis; Jin X designed the study and wrote the manuscript; Liu J and Jin X contribute equally as the first author.

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Data sharing statement: No additional data are available.

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Abstract

AIM: To investigate the expression pattern of plasma long noncoding RNAs (lncRNAs) in Chrohn's disease (CD) patients.

METHODS: Microarray screening and qRT-PCR verification of lncRNAs and mRNAs were performed in CD and control subjects, followed by hierarchy clustering, GO and KEGG pathway analyses. Significantly dysregulated lncRNAs were categorized into subgroups of antisense lncRNAs, enhancer lncRNAs and lincRNAs. To predict the regulatory effect of lncRNAs on mRNAs, a CNC network analysis was performed and cross linked with significantly changed lncRNAs. The overlapping lncRNAs were randomly selected and verified by qRT-PCR in a larger cohort.

RESULTS: Initially, there were 1211 up-regulated and 777 down-regulated lncRNAs as well as 1020 up-regulated and 953 down-regulated mRNAs after microarray analysis; a heat map based on these results showed good categorization into the CD and control groups. GUSBP2 and AF113016 had the highest fold change of the up- and down-regulated lncRNAs, whereas TBC1D17 and CCL3L3 had the highest fold

change of the up- and down-regulated mRNAs. Six (SNX1, CYFIP2, CD6, CMTM8, STAT4 and IGFBP7) of 10 mRNAs and 8 (NR_033913, NR_038218, NR_036512, NR_049759, NR_033951, NR_045408, NR_038377 and NR_039976) of 14 lncRNAs showed the same change trends on the microarray and qRT-PCR results with statistical significance. Based on the qRT-PCR verified mRNAs, 1358 potential lncRNAs with 2697 positive correlations and 2287 negative correlations were predicted by the CNC network.

CONCLUSION: The plasma lncRNAs profiles provide preliminary data for the non-invasive diagnosis of CD and a resource for further specific lncRNA-mRNA pathway exploration.

Key words: Crohn's disease; Long noncoding RNA; Inflammatory bowel disease; Plasma; Microarray

Core tip: The pathogenesis of Chrohn's disease (CD) is unclear while increasing evidence supports the involvement of epigenomic regulation. In this study, we jointly used microarray screening and qRT-PCR verification to achieve the plasma specific long noncoding RNAs expression profile of patients with CD and their potential regulation of downstream mRNAs. Our results would provide preliminary data for non-invasive diagnosis of CD and a reservoir for specific lncRNA-mRNA pathway exploration in the future.

Chen D, Liu J, Zhao HY, Chen YP, Xiang Z, Jin X. Plasma long noncoding RNA expression profile identified by microarray in patients with Crohn's disease. *World J Gastroenterol* 2016; 22(19): 4716-4731 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i19/4716.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i19.4716

INTRODUCTION

Crohn's Disease (CD) is a chronic and relapsing inflammatory disease that could affect any part of the intestine. The prevalence of CD is increasing in developing and developed countries, making it a global health care problem and an interesting research area[1,2]. However, the mechanism of CD remains vague; the involvement of genetic predisposition, immune response and environmental factors has been advocated[3]. Although the development of 5-amino-salicyte acid, prednisone and anti-inflammatory reagents has improved CD therapy, their effects are much more alleviative and sometimes are ineffective for refractory CD[4]. Therefore, the development of novel CD therapeutics is urgently needed, and the exploration of CD mechanisms is of clinical importance.

Recent genome-wide association studies (GWAS) identified novel susceptibility genes for CD[5], highlighting the important role of genomic factors. However, the majority of these studies focused on protein-coding genes and neglected the noncoding RNAs (ncRNAs) that were previously regarded as junk RNA or transcript noises[6]. With the development of high-throughput technologies, huge numbers of ncRNAs were identified, and many ncRNAs have been shown to be involved in physiological processes that maintain cellular and tissue homeostasis[7-9]. Generally, ncRNAs could be categorized into small ncRNAs (< 200 nt), such as microRNAs (miRNAs), and long noncoding RNAs (lncRNAs, > 200 nt). Research on lncRNAs has increased in recent years, showing their potency in regulating protein coding genes at the level of chromatin remodeling, transcriptional control and post-transcriptional processes[10].

A recent study revealed that lncRNAs play a pivotal role in immune function regulation and the progression of autoimmune related diseases, including CD[11]. An in-depth study by Mirza *et al*[12] reported the transcriptomic landscape of lncRNAs in inflammatory bowel disease (IBD). Furthermore, Qiao *et al*[13] identified the increased lncRNA DQ786243 level in CD patients and its effect on the function of regulatory T lymphocytes through changing CREB and Foxp3 levels. However, although evidence for plasma lncRNAs as noninvasive diagnostic biomarkers has accumulated[14,15], none has been reported in CD. Therefore, we conducted microarray screening, qRT-PCR verification and bioinformatics analysis of plasma lncRNAs and mRNAs from CD patients, aiming to provide preliminary data for noninvasive CD diagnosis and investigations into the underlying mechanism of CD.

MATERIALS AND METHODS

Ethics statement

The protocol on human beings was approved by the institutional review board of the First Affiliated Hospital of Zhejiang University and conducted in accordance with the Declaration of Helsinki. The study design and manuscript preparation fully followed the guidelines from the STROBE statement. Written consent was obtained before beginning the study.

Patient sample preparation

CD patients (n = 12) were selected when first diagnosed in the Department of Gastroenterology, The First Affiliated Hospital of Zhejiang University between January 2013 and December 2014. The diagnosis of CD was based on endoscopy manifestations and biopsy, as adopted by the Asia-pacific consensus, with preclusion of intestinal tuberculosis, ulcerative colitis, Bechet's disease and ischemic colitis[16]. To reduce the bias caused by different severities and extents of disease, we narrowed our selection to severe CD with

Table 1 Clinical characteristics and laboratory tests of enrolled subjects

	CD (n = 12)	Control (n = 12)	P value
Gender (M/F)	6/6	7/5	> 0.050[1]
Age (yr)	37.8 ± 14.6	42.8 ± 14.8	0.420
BMI (kg/m^2)	20.8 ± 1.8	23.3 ± 1.4	0.001
CRP (mg/L)	47.3 ± 6.9	4.4 ± 2.1	< 0.001
WBC (10E9/L)	11.6 ± 1.6	5.7 ± 0.9	< 0.001

[1]$\chi^2 = 0.17$.

small intestine involvement and related comorbidities (3 aphthous, 3 perianal abscess, 2 anal fistula and 2 arthralgia). The CD severity degree was assessed based on Harvey-Bradshaw index (HBI) and HBI > 9 was regarded as severe[17]. In CD patients, the average HBI was 11.3. Control subjects (n = 12) were enrolled from healthy volunteers without any health problems during their health checkups at our hospital during the same period. The authors had access to identifying information during or after data collection (Table 1). In this study, 3 of 12 subjects were randomly chosen from each group for microarray analysis, and ensuing qRT-PCR verification of significantly dysregulated lncRNAs and mRNAs was performed for the whole group. All blood samples were collected in a separate vacuum tube and sequentially centrifuged at 3000 rpm for 10 min and at 12000 rpm for 10 min. The cell-free plasma from supernatant was then stored at -80 ℃ for further analysis.

RNA isolation and quality control

Total RNA was isolated from each plasma sample by separately mixing the sample with Polyacryl Carrier (MRC, OH, United States), TRIzol reagent (Invitrogen, Carlsbad, CA, United States) and chloroform, according to the manufacturer's protocol. RNA purification was routinely performed with an RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA quantity was measured with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and RNA quality was tested with an Agilent 2100 Bioanalyzer (Agilent Technologies).

Microarray analysis and computational analysis

A human 8 × 60 KLncRNA/mRNAV3.0 microarray (Arraystar, Rockville, Maryland, United States) containing 30586 human lncRNAs and 26109 protein-coding transcripts was used in our study. Each transcript was represented using 1-5 probes to improve the statistical confidence. Generally, sample labeling and array hybridization were performed (Supplementary method) according to the One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technology, Santa Clara, CA, United States), and Agilent Feature Extraction software (version 11.0.1.1) was used to analyze the acquired array images. The quantile normalization and subsequent data processing were performed with the GeneSpring GX v12.1 software package (Agilent Technologies). Differentially expressed lncRNAs and mRNAs with statistical significance between the two groups were identified through a paired t-test (P < 0.05), multiple hypothesis testing (FDR < 0.05) and fold change filtering (⩾ 2.0 or ⩽ 0.5). Further hierarchical clustering was performed to visualize numerical changes of lncRNAs and samples. The lncRNAs expression data have been deposited into Gene Expression Omnibus (GEO) under accession number GSE75459.

Based on the microarray data, the significantly differentially expressed lncRNAs were further categorized into antisense_LncRNAs, enhancer_LncRNAs and LincRNAs according to their potential effects and associations with downstream mRNAs. For the mRNA analysis, Gene Ontology (GO) that describes genes and gene products in any organism was used, covering the domains of Biological Process (BP), Cellular Component (CC) and Molecular Function (MF). Pathway analysis was also carried out for a functional analysis of mapping genes to KEGG pathways. Fisher's exact/χ^2 test and FDR were jointly used for significance detection. The P-value denotes the significance of GO term and Pathway correlated to the conditions. The lower the P-value, the more significant the Pathway and the GO Term. The FDR indicates the false discovery rate; a smaller FDR indicates smaller error in judging the P-value.

Quantitative real-time polymerase chain reaction validation

The total RNA isolated from the CD and control groups was reverse transcribed using a PrimeScript RTreagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Dalian, China) in accordance with the manufacturer's instructions. U6 snRNA was amplified as a normalization control, and the relative amount of each lncRNA/mRNA to U6 RNA was calculated using the equation $2^{-\Delta CT}$, where $\Delta CT = C_{TmiRNA} - C_{Tu6}$. Based on combinational consideration of the fold change, raw data, FDR, P-value and clinical manifestation reported by previous studies, 10 mRNAs were selected for quantitative real-time polymerase chain reaction (qRT-PCR) verification (Table S1) with a SYBR Green PCR kit (TaKaRa), with 3 replicated each. The same procedure was performed on 14 lncRNAs for verification (Table S1); these lncRNAs were selected from the overlap between the CNC network predications and the lncRNAs microarray data, with further preclusion of fold change < 2 and raw data density < 200.

Co-expression network construction and statistics

The co-expression network of lncRNA-mRNA was constructed based on the correlation between significantly differentially expressed lncRNAs and mRNAs, as previously reported[18]. In the network, a pink node represents a significantly expressed mRNA, and a blue node represents the related

Table 2 Top 10 dysregulated long noncoding RNAs and mRNAs in Chrohn's disease patients

Gene name	Transcript	Fold change
Up-regulated lncRNAs		
GUSBP2	ENST00000466668	626.49
RP5-968D22.1	ENST00000422548	444.43
RP11-68L1.2	ENST00000502712	324.23
RP11-428F8.2	ENST00000425364	245.98
GAS5-AS1	NR_037605	236.81
RP11-923I11.5	ENST00000562996	196.25
DDX11-AS1	NR_038927	192.07
XLOC_005955	TCONS_00014043	175.81
XLOC_005807	TCONS_00012771	87.76
AC009133.20	ENST00000569039	82.92
Down-regulated lncRNAs		
AF113016	uc001ody.3	481.03
ALOX12P2	ENST00000575787	298.81
AGSK1	uc010bmo.1	208.70
CTC-338M12.3	ENST00000509252	172.74
AC064871.3	ENST00000413954	96.13
RP11-510H23.3	ENST00000431104	77.63
LOC729678	uc011dhd.2	69.95
XLOC_010037	TCONS_00020749	66.82
LOC283761	NR_027074	58.70
XLOC_013142	TCONS_00027621	45.60
Up-regulated mRNAs		
TBC1D17	NM_024682	488.98
GALNT8	NM_017417	303.23
DENND1A	NM_024820	301.75
VANGL1	NM_001172411	247.83
VPS29	NM_057180	189.17
EHD1	NM_006795	132.08
FAM84A	NM_145175	121.69
SAA4	NM_006512	105.50
ZNF33A	NM_006974	101.84
GKN1	NM_019617	91.51
Down-regulated mRNAs		
CCL3L3	NM_001001437	994.63
BGLAP	NM_199173	501.53
SLC51B	NM_178859	499.44
BAG4	NM_004874	134.54
MAU2	NM_015329	115.54
TSNARE1	NM_145003	106.10
DIXDC1	NM_033425	93.11
ZBTB25	NM_006977	82.42
CMTM8	NM_178868	75.71
ANXA1	NM_000700	69.07

The top 10 dysregulated lncRNAs and mRNAs are listed based on significantly differentially expressed lncRNAs and mRNAs ($P < 0.05$) and the fold change.

lncRNA. Moreover, the red solid line represents a direct connection of a positive correlation between specific lncRNAs and mRNAs, and the green line represents a direct connection of a negative correlation. SPSS (version 16.0, Chicago, IL, United States) was used for statistical analyses. The data are expressed as the mean ± SD. Variables of the microarray and qRT-PCR data between the two groups were compared by Student's *t*-test. In the microarray results, a fold change of lncRNAs/mRNAs $\geqslant$ 2.0 was chosen for further analysis, and $P < 0.05$ was considered statistically significant.

RESULTS

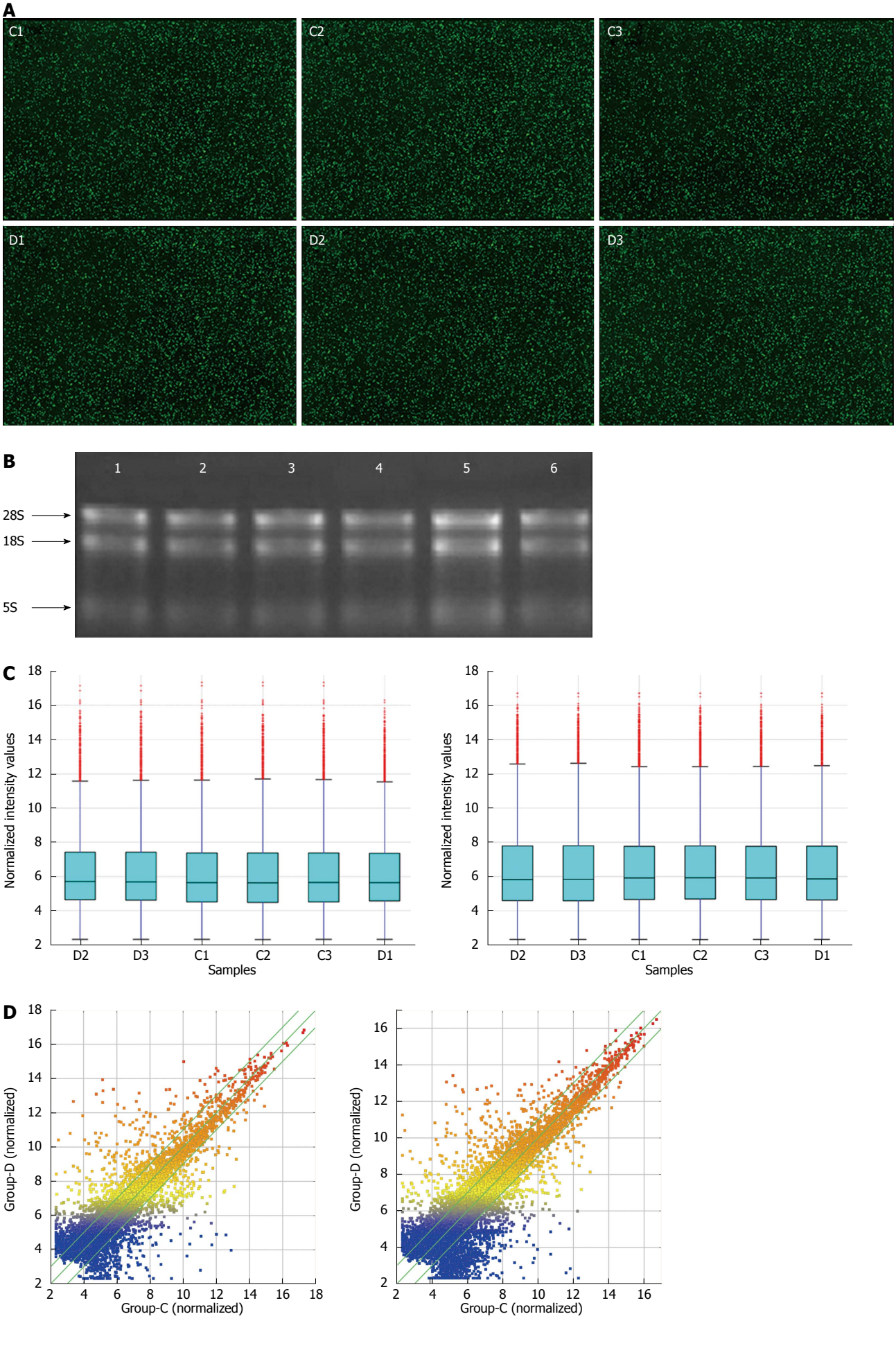
Expression pattern of lncRNAs and mRNAs in CD

Compared with the control group, the BMI was significantly lower and the WBC and CRP were significantly higher in CD patients (Table 2). A microarray analysis of lncRNAs and mRNAs was carried out in randomly selected subjects. As jointly evaluated by heat map, box plot, scatter plot and volcano plot, the differential expression of lncRNAs and mRNAs was well categorized in the CD and control groups with good RNA quality and microarray image control (Figure 1). Compared with the control group, there were 1211 up-regulated and 777 down-regulated lncRNAs in the CD group (fold change $\geqslant$ 2.0, $P < 0.05$; Table S2). Moreover, there were 1020 up-regulated and 953 down-regulated mRNAs (fold change $\geqslant$ 2.0, $P < 0.05$; Table S3). The top 10 dysregulated lncRNAs and mRNAs are summarized in Table 2.

Computational analysis of significantly dysregulated lncRNAs and mRNAs

To narrow the large number of lncRNAs retrieved from the microarray data, we further carried out lncRNAs subgroup analyses. Based on the association between an lncRNA and its nearby mRNA, lncRNAs were categorized into antisense lncRNAs, enhancer lncRNAs and lincRNAs, providing a more accurate source for further functional study (Table S4). Briefly, among the 15 antisense lncRNAs, up-regulated ENST00000569039 had the highest fold change of 82.92, targeting the nearby gene NM_001042539 (myc-associated zinc finger protein isoform 2); the down-regulated ENST00000555407 had the highest fold change of 4.97, targeting the nearby gene NM_001085471 (forkhead box protein N3 isoform 1). Of the 81 enhancer lncRNAs, up-regulated ENST00000422548 had the highest fold change of 444.43, targeting the nearby gene ENST00000367818 [chemokine (C motif) ligand 1], and the down-regulated ENST00000427085 had the highest fold change of 24.29, targeting the nearby gene ENST00000534062 (retrotransposon-like 1). Finally, in the 161 lincRNAs, the up-regulated TCONS_00027580 had the highest fold change of 54.09, targeting the nearby gene NM_001102599 (carcinoembryonic antigen-related cell adhesion molecule 20 isoform 4L precursor), and the down-regulated TCONS_00027621 had the highest fold change of 45.60, targeting the nearby gene NM_001164309 (zinc finger protein 415 isoform 2).

The top 10 dysregulated GO processes of each subgroup (BP, CC and MF) are presented in Figures 2 and 3. Because the GO manifestations in up- and down-regulated mRNAs varied, we analyzed them separately. In the up-regulated mRNAs as shown in Figure 2, the largest GO processes included single-organism process, neurogenesis and negative


A
C1
C2
C3
D1
D2
D3
B
1
2
3
4
5
6
28S
18S
5S
C
Normalized intensity values
Samples
D2
D3
C1
C2
C3
D1
D
Group-D (normalized)
Group-C (normalized)

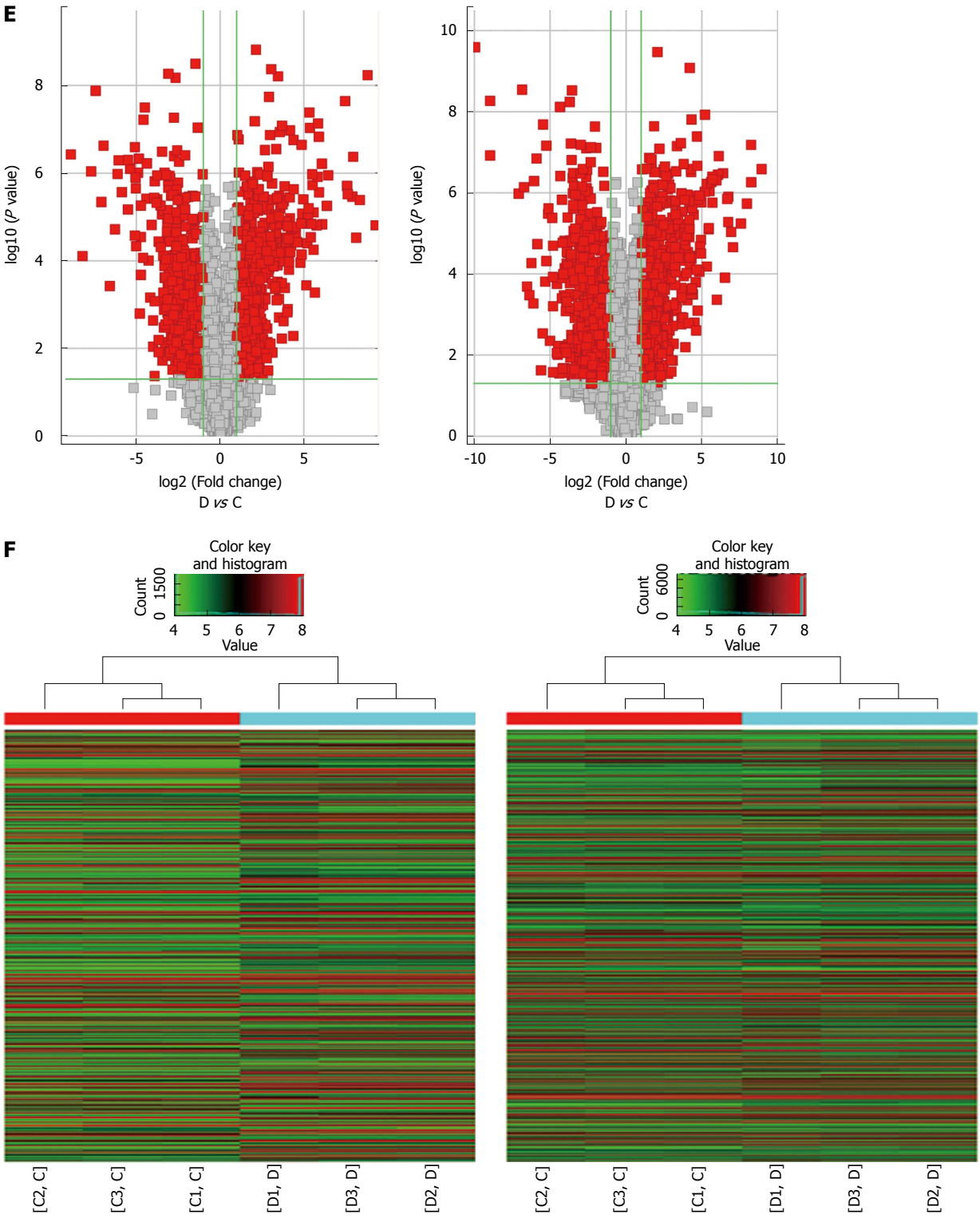
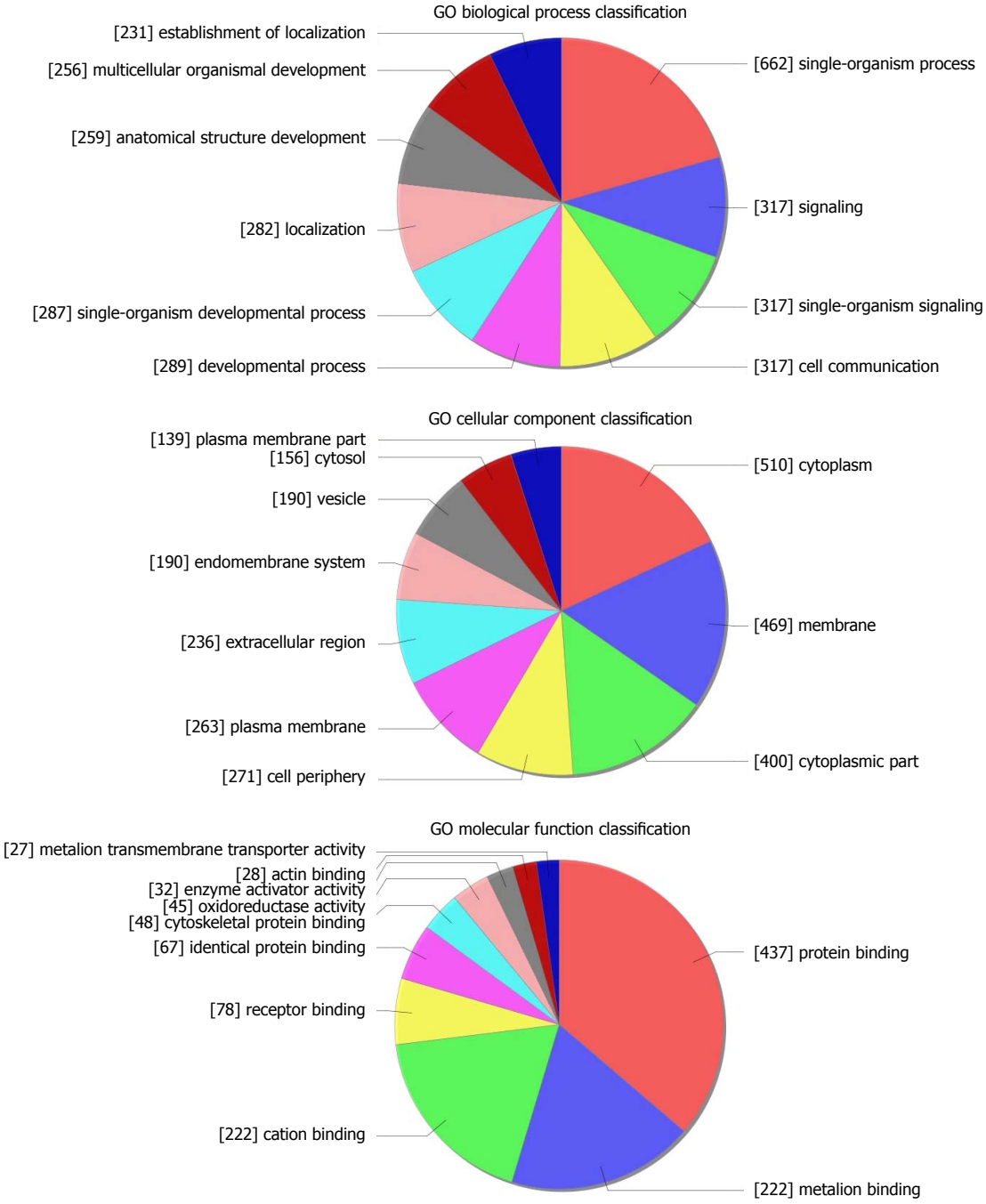
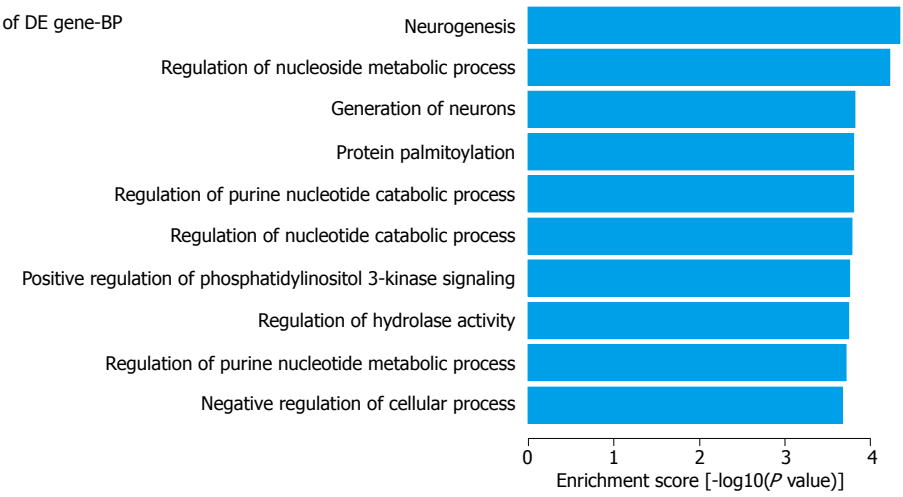


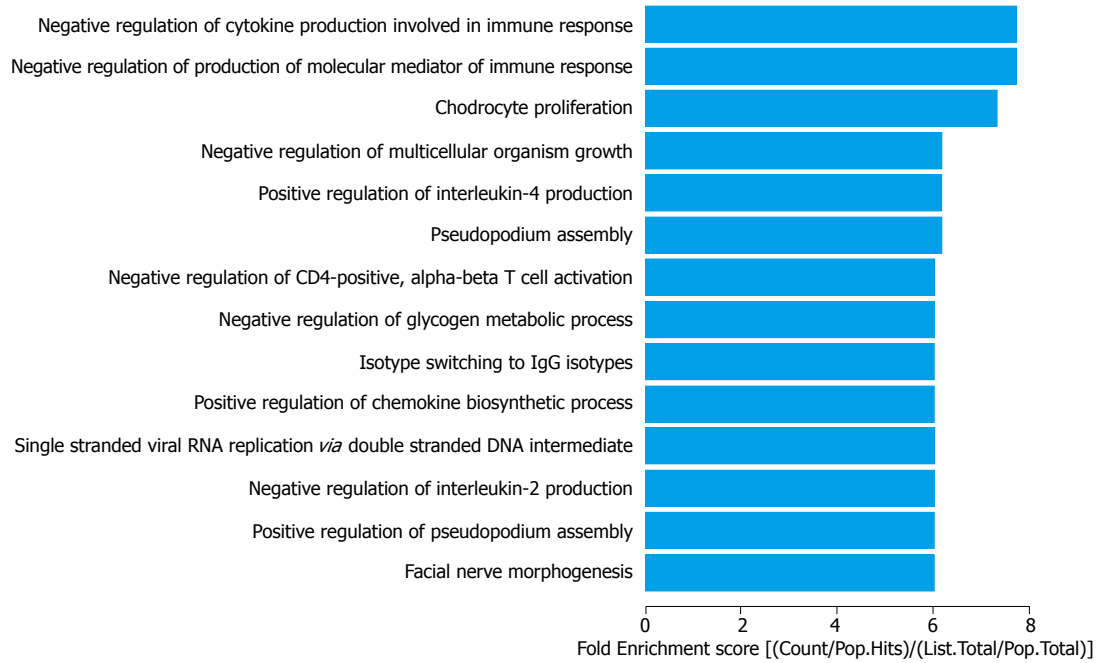
Figure 1 Raw image, RNA quality and bioinformatics analysis of differentially expressed long noncoding RNAs and mRNAs in Chrohn's disease patients. A: Raw image of the microarray analysis. The green dot on the black background represents a single lncRNA or mRNA. C1-C3 were control samples, and D1-D3 were CD samples; B: RNA elecotrophoretogram showing good RNA quality (1-3 were control, and 4-6 were CD); C: Box plot visualizing the lncRNA (left panel) and mRNA (right panel) expression variations; D, E: Scatter plot and volcano plot showing the distributions of lncRNAs (left panel) and mRNAs (right panel) in a more direct way. After normalization, the distributions of the log2 ratios among samples were nearly the same. The values of the X- and Y-axes in the scatter plot were the averaged normalized signal values of the group (log2 scaled). The green lines in the scatter plot and volcano plot represent the default significant fold change (2.0); F: Hierarchical cluster analysis of microarray data assessing the significant expression of lncRNAs (left panel) and mRNAs (right panel) between the CD and control groups. Red and green denote high and low expression, respectively. Each RNA is represented by a single row of colored boxes, and each sample is represented by a single column.

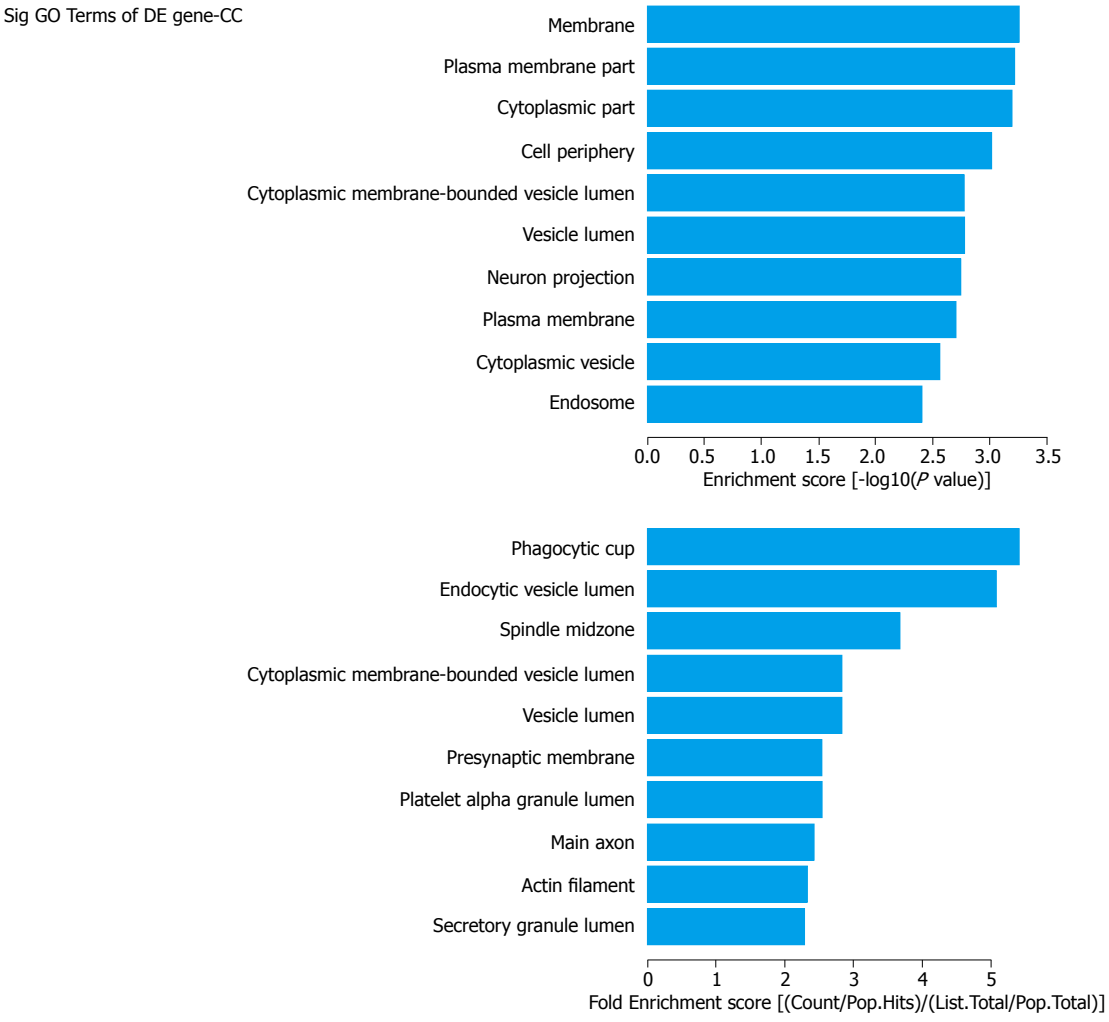
A


GO biological process classification
[231] establishment of localization
[256] multicellular organismal development
[259] anatomical structure development
[282] localization
[287] single-organism developmental process
[289] developmental process
[662] single-organism process
[317] signaling
[317] single-organism signaling
[317] cell communication
GO cellular component classification
[139] plasma membrane part
[156] cytosol
[190] vesicle
[190] endomembrane system
[236] extracellular region
[263] plasma membrane
[271] cell periphery
[510] cytoplasm
[469] membrane
[400] cytoplasmic part
GO molecular function classification
[27] metalion transmembrane transporter activity
[28] actin binding
[32] enzyme activator activity
[45] oxidoreductase activity
[48] cytoskeletal protein binding
[67] identical protein binding
[78] receptor binding
[222] cation binding
[437] protein binding
[222] metalion binding

B


Sig GO Terms of DE gene-BP
Neurogenesis
Regulation of nucleoside metabolic process
Generation of neurons
Protein palmitoylation
Regulation of purine nucleotide catabolic process
Regulation of nucleotide catabolic process
Positive regulation of phosphatidylinositol 3-kinase signaling
Regulation of hydrolase activity
Regulation of purine nucleotide metabolic process
Negative regulation of cellular process
0
1
2
3
4
Enrichment score [-log10(P value)]


Negative regulation of cytokine production involved in immune response
Negative regulation of production of molecular mediator of immune response
Chodrocyte proliferation
Negative regulation of multicellular organism growth
Positive regulation of interleukin-4 production
Pseudopodium assembly
Negative regulation of CD4-positive, alpha-beta T cell activation
Negative regulation of glycogen metabolic process
Isotype switching to IgG isotypes
Positive regulation of chemokine biosynthetic process
Single stranded viral RNA replication *via* double stranded DNA intermediate
Negative regulation of interleukin-2 production
Positive regulation of pseudopodium assembly
Facial nerve morphogenesis
0
2
4
6
8
Fold Enrichment score [(Count/Pop.Hits)/(List.Total/Pop.Total)]


Sig GO Terms of DE gene-CC
Membrane
Plasma membrane part
Cytoplasmic part
Cell periphery
Cytoplasmic membrane-bounded vesicle lumen
Vesicle lumen
Neuron projection
Plasma membrane
Cytoplasmic vesicle
Endosome
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
Enrichment score [-log10(*P* value)]
Phagocytic cup
Endocytic vesicle lumen
Spindle midzone
Cytoplasmic membrane-bounded vesicle lumen
Vesicle lumen
Presynaptic membrane
Platelet alpha granule lumen
Main axon
Actin filament
Secretory granule lumen
0
1
2
3
4
5
Fold Enrichment score [(Count/Pop.Hits)/(List.Total/Pop.Total)]

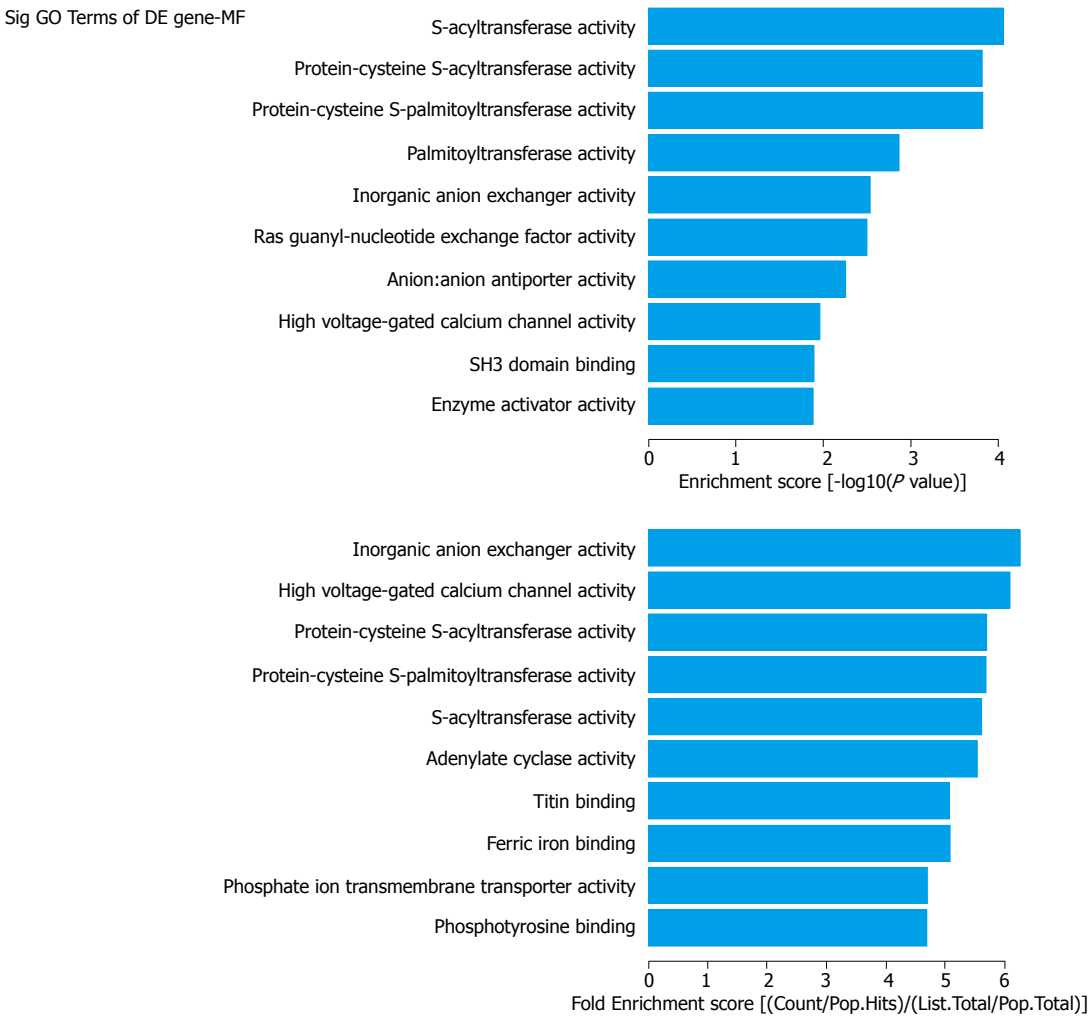


Figure 2 Gene ontology analysis of up-regulated mRNAs. A: The GO analysis includes classification of biological process (BP), cellular component (CC) and molecular function (MF); B: The summaries of significant GO terms (BP, CC and MF) of differentially expressed genes are shown in the up and down panels according to the values in the enrichment score and the fold enrichment.

regulation of cytokine production involved in immune response in BP; cytoplasm, membrane and phagocytic cup in CC; protein binding, S-acyltransferase activity and inorganic anion exchanger activity in MF, according to the different algorithms of routine classification, enrichment score and fold enrichment. Similarly, the largest GO processes of the down-regulated mRNAs were cellular process, presentation of exogenous peptide antigen *via* MHC class I TAP dependent, antigen processing and presentation of endogenous peptide antigen in BP; cell part, extracellular vesicular exosome and MHC class I protein complex in CC and binding, protein binding and RAGE receptor binding in MF (Figure 3).

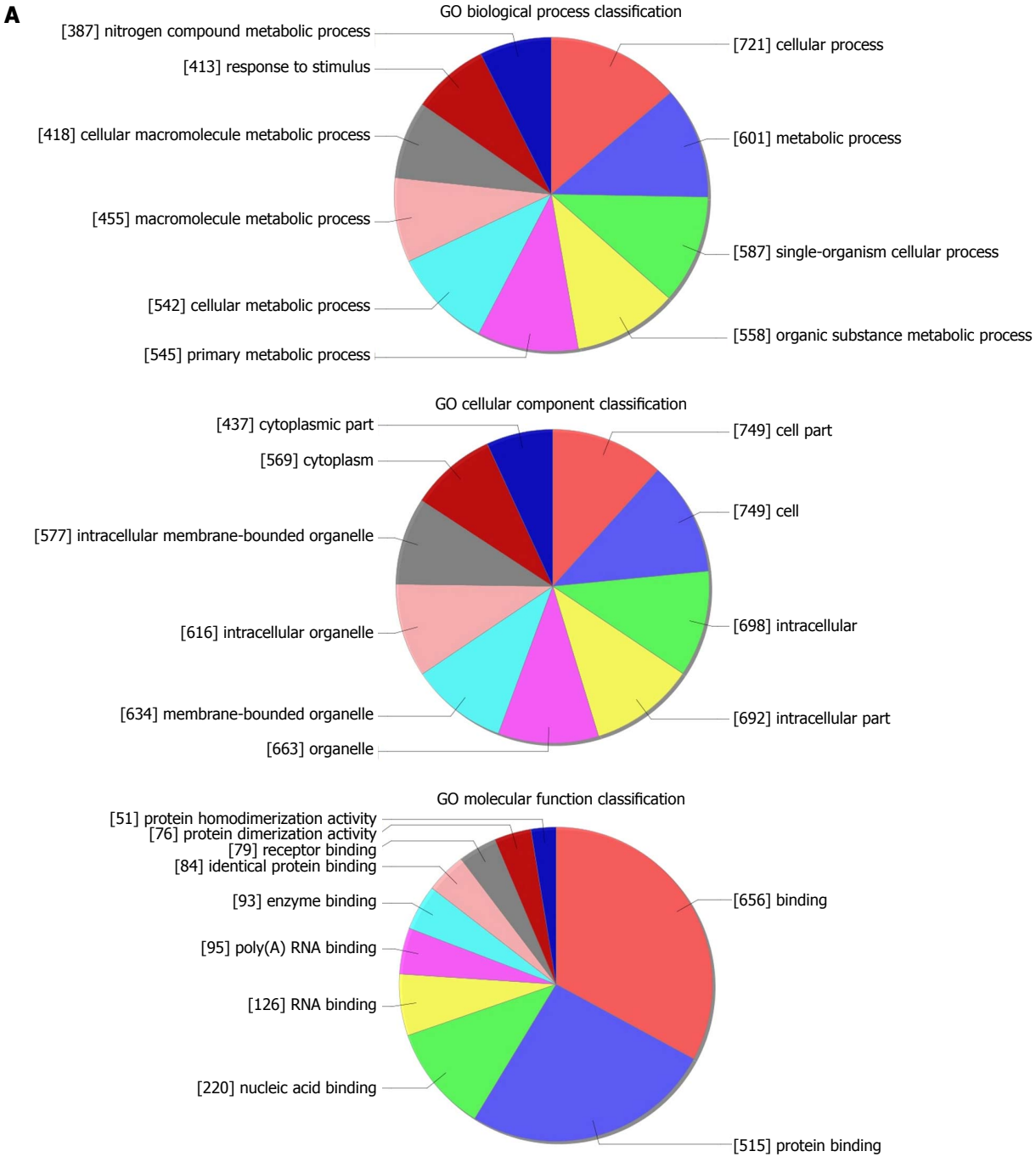
As further shown in Table S5, through the KEGG pathway analysis, 37 gene pathways were found to be targeted in up-regulated mRNAs; the top 3 processes were dilated cardiomyopathy, endocytosis and the estrogen signaling pathway (Figure 4A). More importantly, the IBD process itself was at the sixth of the top 10 pathways according to the enrichment score. Similarly, 32 gene pathways were found in down-regulated mRNAs; the top 3 processes were Huntington's disease, proteasome and oxidative phosphorylation (Figure 4B).

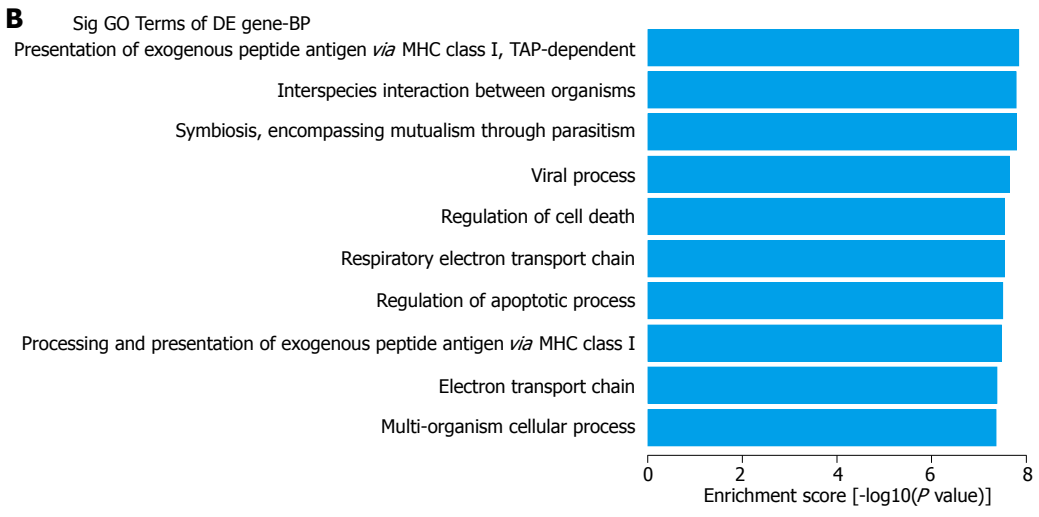
Downstream mRNAs selection and qRT-PCR verification

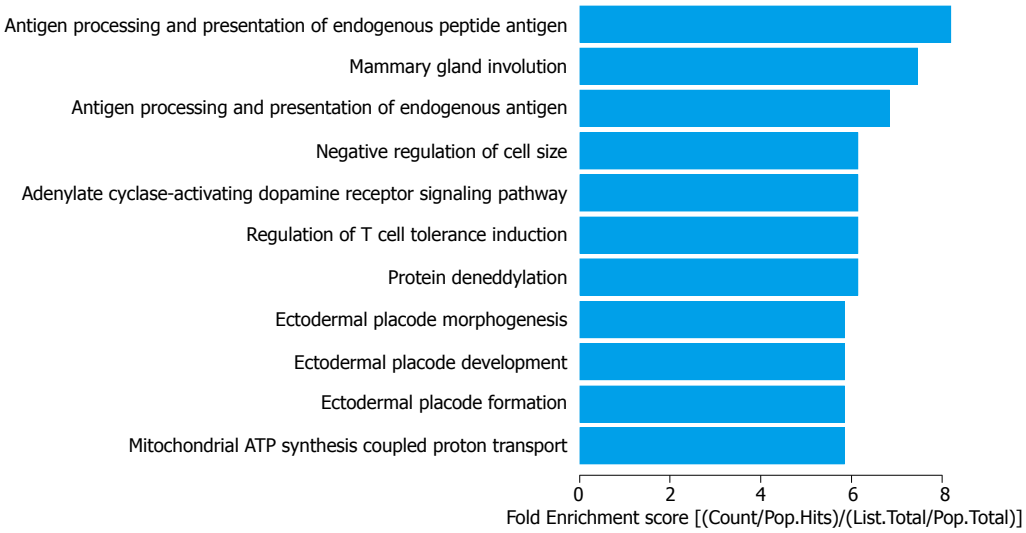
Based on the high normalized intensity in the raw data, high fold change, significant *P*-value and clinical meanings, we selected 10 mRNAs (SNX1, CYFIP2, CD6, CMTM8, AURKB, BGLAP, STAT4, WNT4, IGFBP7 and TGFβ-2) for qRT-PCR verification. As shown in Figure 5A, six mRNAs (SNX1, CYFIP2, CD6, CMTM8, STAT4 and IGFBP7) showed the same change tendency between the microarray and qRT-PCR results with statistical significance. AURKB and WNT4 showed the opposite change between the microarray and qRT-PCR results without statistical significance.

CNC network construction and predicted lncRNAs verification by qRT-PCR

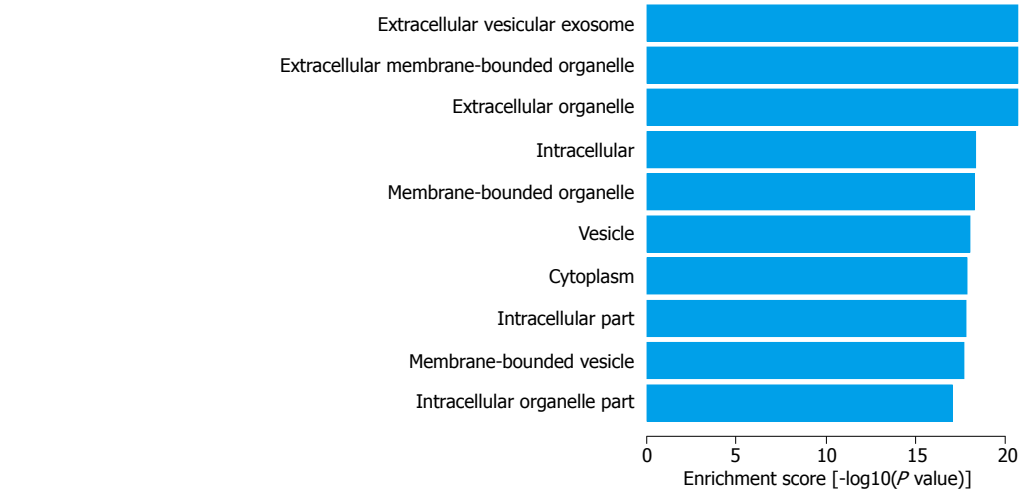
Because lncRNAs participate in the regulation of gene expression in transcriptional, epigenetic and posttranscriptional stages, it is plausible that certain

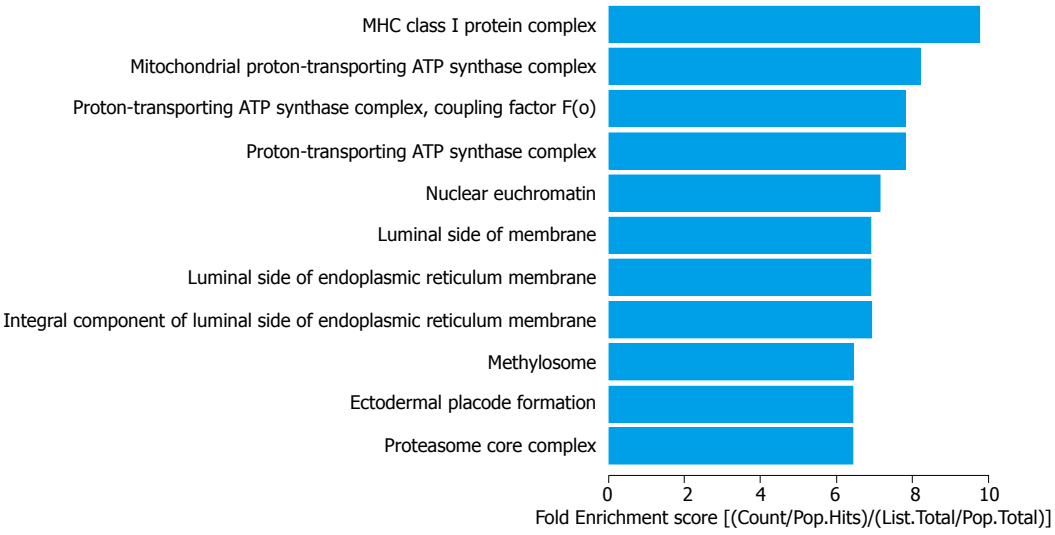

A
GO biological process classification
[387] nitrogen compound metabolic process
[413] response to stimulus
[418] cellular macromolecule metabolic process
[455] macromolecule metabolic process
[542] cellular metabolic process
[545] primary metabolic process
[721] cellular process
[601] metabolic process
[587] single-organism cellular process
[558] organic substance metabolic process
GO cellular component classification
[437] cytoplasmic part
[569] cytoplasm
[577] intracellular membrane-bounded organelle
[616] intracellular organelle
[634] membrane-bounded organelle
[663] organelle
[749] cell part
[749] cell
[698] intracellular
[692] intracellular part
GO molecular function classification
[51] protein homodimerization activity
[76] protein dimerization activity
[79] receptor binding
[84] identical protein binding
[93] enzyme binding
[95] poly(A) RNA binding
[126] RNA binding
[220] nucleic acid binding
[656] binding
[515] protein binding


B
Sig GO Terms of DE gene-BP
Presentation of exogenous peptide antigen *via* MHC class I, TAP-dependent
Interspecies interaction between organisms
Symbiosis, encompassing mutualism through parasitism
Viral process
Regulation of cell death
Respiratory electron transport chain
Regulation of apoptotic process
Processing and presentation of exogenous peptide antigen *via* MHC class I
Electron transport chain
Multi-organism cellular process
0
2
4
6
8
Enrichment score [-log10(*P* value)]



Sig GO Terms of DE gene-CC





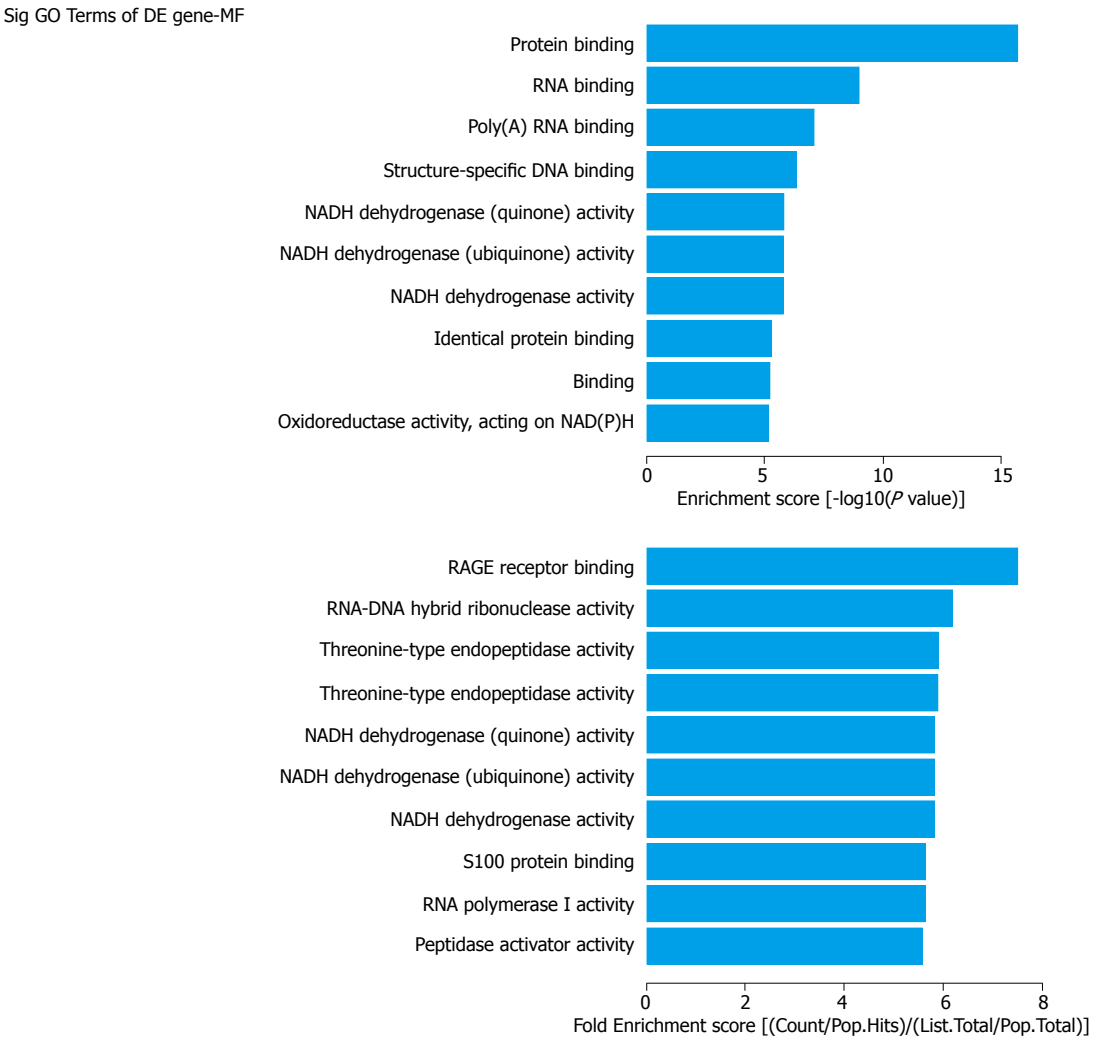


Figure 3 Gene ontology analysis of down-regulated mRNAs. A: The GO analysis includes classification of biological process (BP), cellular component (CC) and molecular function (MF); B: The summaries of significant GO terms (BP, CC and MF) of differentially expressed genes are shown in the up and down panels according to the values in the enrichment score and the fold enrichment.

lncRNAs are involved in CD pathogenesis. Based on the six qRT-PCR verified mRNAs, we predicted 1358 potential lncRNAs with 2697 positive correlations and2287 negative correlations between mRNA and lncRNAs by CNC network construction (Figure S1 and Table S6). After cross-linking between CNC predicted lncRNAs and lncRNAs microarray results, we selected 14 lncRNAs (NR_033913, NR_073047, NR_038927, NR_038218, NR_036512, NR_072994, NR_049759, NR_046052, NR_033951, NR_045408, NR_038377, NR_015413, NR_039976 and NR_038345) for qRT-PCR verification based on the selection criteria used for mRNAs. As shown in Figure 5B, 8 lncRNAs (NR_033913, NR_038218, NR_036512, NR_049759, NR_033951, NR_045408, NR_038377 and NR_039976) showed the same change tendency between the microarray and qRT-PCR results with statistical significance. Two lncRNAs (NR_073047 and NR_015413) showed the opposite change tendency without statistical significance. Four lncRNAs (NR_038927, NR_072994, NR_046052 and NR_038345) showed the same change tendency as in microarray results, but the results did not reach statistical significance (See detailed qRT-PCR and microarray results in Table S7).

DISCUSSION

CD is an important subtype of IBD with characteristics of intestinal full-thickness lesions and severe complications including perforation, fistula formation, malnutrition and carcinogenesis. Currently, next-generation sequencing and high-density microarrays have provided novel methods for CD study. For example, the NOD2/CARD15 gene mutation was identified to be associated with a CD phenotype[19], whereas a novel CD locus mapping to a gene desert on 5p 13.1 was also reported[20]. Nevertheless, according to the recent meta-analysis of GWAS studies, the number of confirmed genetic loci associated with CD was 150, although an overwhelming majority of these loci are located in noncoding regions[5], suggesting the importance of ncRNAs in CD research. A recent study showed that the loss of endogenous intestinal

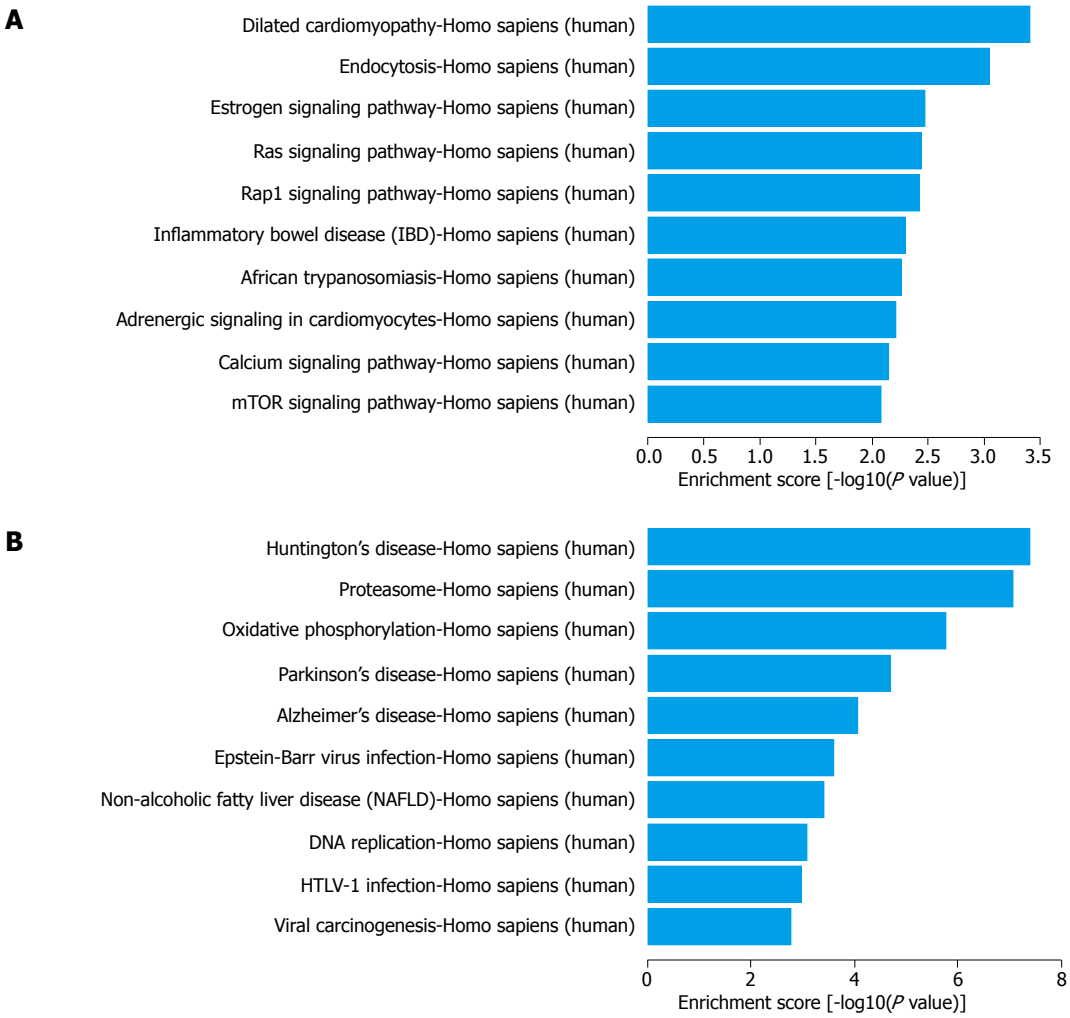


Figure 4 Top 10 KEGG pathways in up- (A) and down-regulated (B) mRNAs.

miRNAs caused impairment of epithelial barrier function, resulting in acute inflammation[21]. Further accumulating data supported an active role of miRNAs in the pathogenesis of IBD[22]. However, research into lncRNAs, another subgroup of ncRNAs with ability in binding protein, RNA and DNA, in CD has been rarely reported.

In this study, we used microarray screening and qRT-PCR verification to obtain the profile of plasma lncRNAs in carefully selected CD patients. We identified a total of 1988 and 2993 dysregulated lncRNAs and mRNAs between the CD and control groups; the numbers in our study were much higher than 450 lncRNAs and 1100 mRNAs from Mirza's report[12]. This difference might be due to the relatively lower sample size of our study, which makes the data more dispersed. Moreover, there were no overlap of dysregulated lncRNAs between our results and the top 10 dysregulated lncRNAs we extracted from Mirza's paper, except one lncRNA - DIO3OS that was up-regulated in our but down-regulated in their study. One possible explanation might be the secretion of DIO3OS from intestinal tissue to circulation, which needs further investigation. The intrinsic difference between our and Mirza's results is the source of the lncRNAs. We reported, for the first time, the plasma lncRNAs in CD patients. If we could enlarge sample size and narrow down the scale of plasma lncRNAs, this method would become a good candidate for the non-invasive diagnosis of CD. Circulating lncRNAs (serum and plasma) have already been used as non-invasive biomarkers for liver cancer[23], breast cancer[24] and lung cancer[25], implying their wide application potential. It is theoretically plausible to use plasma lncRNAs because they are quite stable when included in lipid or lipoprotein vesicles in the circulation. A recent study showed that lncRNAs might be protected by exosomes in blood[26]. Nevertheless, the detailed secretion mechanisms of lncRNAs remain vague.

The application of bioinformatics is pivotal for in-depth analyses of huge data from microarray results. For lncRNAs, we used subgroup analyses based on the category of antisense lncRNAs, enhancer lncRNAs and lincRNAs, according to the effect and gene locus of the lncRNAs. For mRNAs, we combined the GO and KEGG pathway for enrichment analysis. In the GO analysis, we found that the largest portion of mRNAs was located in the cytoplasm and was involved in

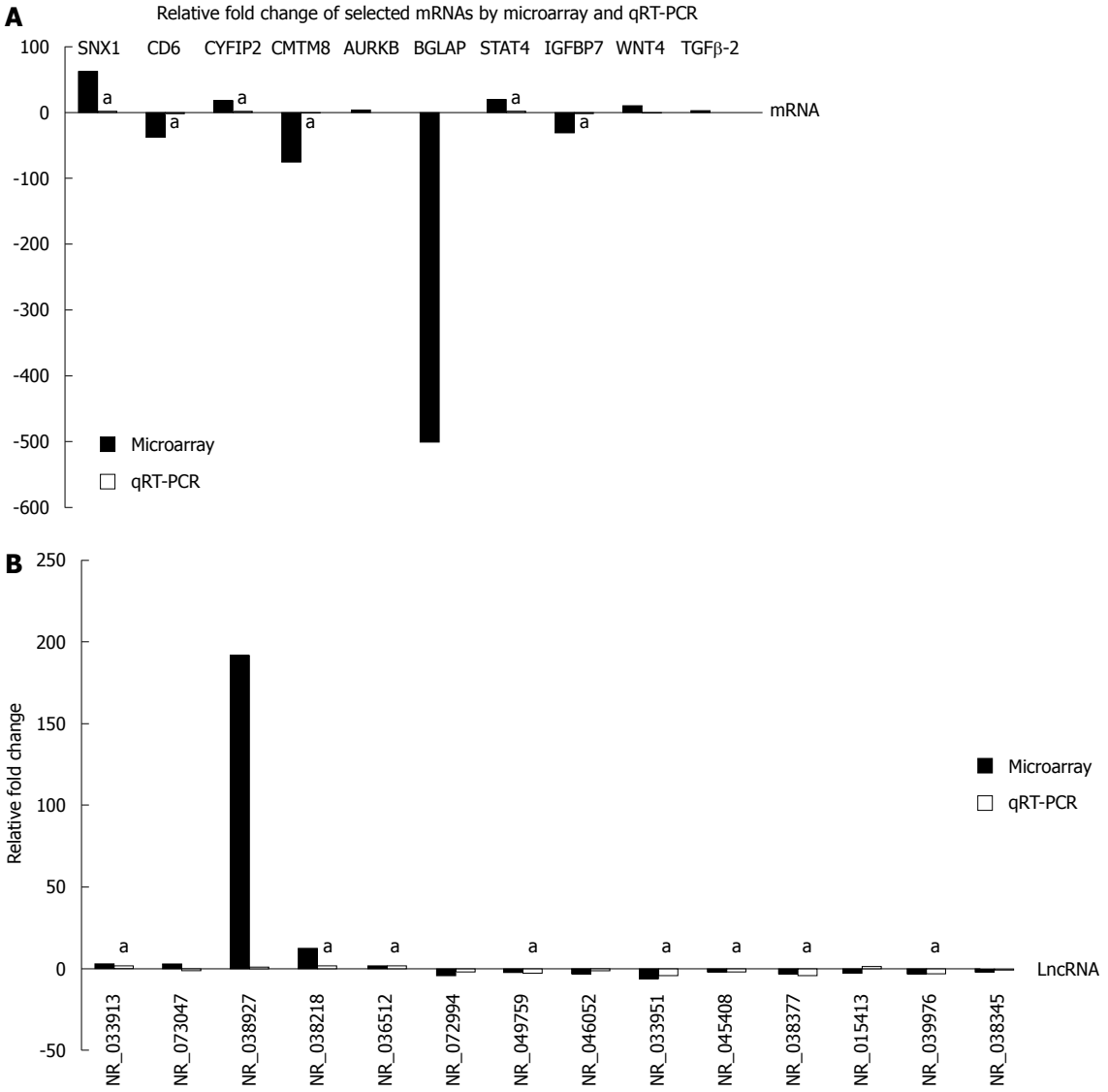


Figure 5 Relative fold changes of lncRNAs and mRNAs by microarray and qRT-PCR. The column upwards indicates up-regulation, and downwards indicates down-regulation. [a]*P* < 0.05, microarray *vs* qRT-PCR. A: mRNA data; B: LncRNA data.

single-organism process and protein binding activity. In the KEGG pathways, we found IBD and oxidative phosphorylationin the list of the top 10 dysregulated pathways. The former finding supported the effectiveness of our study whereas the latter finding revealed the possibility of oxidative stress and energy metabolism in CD pathogenesis.

Of the top 10 up- and down-regulated lncRNAs (Table 2), only GAS5-AS1 was reported to be associated with various cancers[27], leaving a large blank area for further study. Of the top 10 up-regulated mRNAs, VANGL1 is a planar cell polarity component with a developing role in cancer[28], and FAM84A is associated with the enhanced migration of human colon cancer cells[29]. These mRNAs might contribute to the more frequent occurrence of colon cancer in the background of CD. Plasma SAA4 is present mostly in high density lipoproteins[30]. Therefore, the increased expression of SAA4 in CD might imply its potential role in assisting the stable existence of lncRNAs in the circulation. Of the top 10 down-regulated mRNAs, CMTM8 was revealed to induce caspase related apoptosis through a mitochondrial–mediated pathway[31], whereas BAG4 is involved in mitochondrial apoptosis[32], indicating the importance of apoptosis in the aetiology of CD. Moreover, ZBTB25 was found as a novel NF-AT repressor that participates in T-cell development, differentiation and lineage-specific transcription[33], emphasizing the importance of T cell mediated immune imbalance in CD pathogenesis.

Because we listed the top 10 dysregulated lncRNAs and mRNAs according to the relative fold change, there might be some genes that actually have raw data of low density (< 200). Therefore, we first selected 10 mRNAs for qRT-PCR verification based on the joint consideration of the *P*-value (< 0.05), fold change (> 2) and raw data (> 200). Then, we used CNC network analysis with combination of the mRNAs selection criteria and selected 14 lncRNAs for further qRT-PCR verification. Of the finally verified 6 mRNAs, a significantly increased STAT4 level was also reported in previous report[34],

implying its importance in CD. Moreover, CD6 acts as a cell surface receptor and a target for regulating immune responses[35], whereas CYFIP2 is involved in T cell adhesion[36], further emphasizing the importance of T cell mediated immunity in CD. In 8 of 12 verified lncRNAs, NR_049759 is the transcript variant 2 of the coding gene IFITM3 that was up-regulated in colon mucosa of DSS induced mice[37], an animal model of IBD. NR_045408 is the antisense RNA of the coding gene RCNA3 that acts as a tumor suppressor[38], indicating the possibility of the NR_045408-RCNA3 pathway involvement in the occurrence of cancer in CD.

We identified the expression pattern of plasma lncRNAs based on microarray data. Further bioinformatics analyses successfully categorized the subjects into CD and control groups according to the lncRNAs profile, supporting the possibility of using this method as a non-invasive method for CD diagnosis. However, due to the small sample size, the retrieved profile contains many lncRNAs, which might become an obstacle for further application in clinics. Therefore, enlarging the experimental size to narrow the enrolled lncRNAs is urgently needed. Furthermore, because we only focused on severe CD subjects in this study, investigating lncRNAs expression in mild and moderate CD is suggested. Finally, it is better if we can compare the lncRNAs in plasma and intestinal tissue, which may be helpful for the mechanism exploration of CD. For the mRNA data, the GO and KEGG pathways were used to obtain more information. Approximately 60% of the microarray retrieved lncRNAs and mRNAs were verified by qRT-PCR, supporting the effectiveness of microarray screening. Several qRT-PCR verified lncRNAs and mRNAs were related with cancer or T-cell mediated immunity. Therefore, our data also provide a resource for further study of the lncRNA-mRNA pathway in CD pathogenesis.

COMMENTS

Background

Crohn's disease (CD) has been regarded as a chronic and relapsing inflammatory disease that could affect any part of the intestine. The prevalence of CD is increasing in developing and developed countries, making it a global health care problem and an interesting research area.

Research frontiers

The mechanism of CD remains vague; the involvement of genetic predisposition, immune response and environmental factors has been advocated. Currently, with the development of high-throughput technologies, the effect of noncoding RNAs, mainly divided into microRNAs and long noncoding RNAs, has been intensively investigated in CD pathogenesis.

Innovations and breakthroughs

Although evidence for plasma long noncoding RNAs (lncRNAs) as noninvasive diagnostic biomarkers has accumulated, none have been reported in CD. Therefore, we conducted, for the first time, microarray screening, qRT-PCR verification and bioinformatics analysis of plasma lncRNAs and mRNAs from CD patients, aiming to provide preliminary data for noninvasive CD diagnosis and investigations into the underling mechanism of CD.

Applications

Although further verification is needed in a larger independent cohort study, the profile of plasma lncRNAs would provide data for noninvasive diagnosis of CD and the potential lncRNA-mRNA pairs may shed light on the pathogenesis of CD.

Terminology

LncRNAs are a group of RNAs that have the length > 200 nt and do not encodes proteins but exert function of post-transcriptional regulation.

Peer-review

In this work, authors investigated the expression pattern of plasma lncRNAs in CD patients by microarray screening and qRT-PCR verification of lncRNAs and mRNAs, followed by hierarchy clustering, GO and KEGG pathway analysis.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Veterans health administration hepatitis B testing and treatment with anti-CD20 antibody administration

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Abstract

AIM: To evaluate pretreatment hepatitis B virus (HBV) testing, vaccination, and antiviral treatment rates in Veterans Affairs patients receiving anti-CD20 Ab for quality improvement.

METHODS: We performed a retrospective cohort study using a national repository of Veterans Health Administration (VHA) electronic health record data. We identified all patients receiving anti-CD20 Ab treatment (2002-2014). We ascertained patient demographics, laboratory results, HBV vaccination status (from vaccination records), pharmacy data, and vital status. The high risk period for HBV reactivation is during anti-CD20 Ab treatment and 12 mo follow up. Therefore, we analyzed those who were followed to death or for at least 12 mo after completing anti-CD20 Ab. Pretreatment serologic tests were used to categorize chronic HBV (hepatitis B surface antigen positive or HBsAg+), past HBV (HBsAg-, hepatitis B core antibody positive or HBcAb+), resolved HBV (HBsAg-, HBcAb+, hepatitis B surface antibody positive or HBsAb+), likely prior vaccination (isolated HBsAb+), HBV negative (HBsAg-, HBcAb-), or unknown. Acute hepatitis B was defined by the appearance of HBsAg+ in the high risk period in patients who were pretreatment HBV negative. We assessed HBV antiviral treatment and the incidence of hepatitis, liver failure, and death during the high risk period. Cumulative hepatitis, liver failure, and death after anti-CD20 Ab initiation were compared by HBV disease categories and differences compared using the χ^2 test. Mean time to hepatitis peak alanine aminotransferase, liver failure, and death relative to anti-CD20 Ab administration and follow-up were also compared by HBV disease group.

RESULTS: Among 19304 VHA patients who received anti-CD20 Ab, 10224 (53%) had pretreatment HBsAg testing during the study period, with 49% and 43% tested for HBsAg and HBcAb, respectively within 6 mo pretreatment in 2014. Of those tested, 2% (167/10224) had chronic HBV, 4% (326/7903) past HBV, 5% (427/8110) resolved HBV, 8% (628/8110) likely prior HBV vaccination, and 76% (6022/7903) were HBV negative. In those with chronic HBV infection, $\leqslant$ 37% received HBV antiviral treatment during the high risk period while 21% to 23% of those with past or resolved HBV, respectively, received HBV antiviral treatment. During and 12 mo after anti-CD20 Ab, the rate of hepatitis was significantly greater in those HBV positive *vs* negative (P = 0.001). The mortality rate was 35%-40% in chronic or past hepatitis B and 26%-31% in hepatitis B negative. In those pretreatment HBV negative, 16 (0.3%) developed acute hepatitis B of 4947 tested during anti-CD20Ab treatment and follow-up.

CONCLUSION: While HBV testing of Veterans has increased prior to anti-CD20 Ab, few HBV+ patients received HBV antivirals, suggesting electronic health record algorithms may enhance health outcomes.

Key words: Hepatitis B; Hepatitis B reactivation; Anti-CD20 antibody; Rituximab; Lymphoma; Chemotherapy; Hepatitis B antivirals; Vaccination; Veteran

Core tip: Prior to anti-CD20 antibody (Ab) treatment in 2014, 61%-73% of 19304 Veterans had hepatitis B virus (HBV) tests. Of these, 11% tested were positive for hepatitis B surface antigen or core antibody and at risk for reactivation; $\leqslant$ 37% of these HBV+ patients received HBV antivirals during anti-CD20 Ab and follow-up. HBV+ patients had significantly higher hepatitis rates than HBV-. Among pretreatment HBV- patients, about 1 in 300 tested suffered acute hepatitis during anti-CD20 Ab and 12 mo follow-up. Electronic health record algorithms to increase HBV testing, antiviral use and vaccination will likely improve outcomes with anti-CD20 Ab treatment.

Hunt CM, Beste LA, Lowy E, Suzuki A, Moylan CA, Tillmann HL, Ioannou GN, Lim JK, Kelley MJ, Provenzale D. Veterans health administration hepatitis B testing and treatment with anti-CD20 antibody administration. *World J Gastroenterol* 2016; 22(19): 4732-4740 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i19/4732.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i19.4732

INTRODUCTION

In the United States, 4% of the population has

had hepatitis B viral (HBV) infection and 0.3% have chronic hepatitis B (with positive hepatitis B surface antigen)[1,2]. Following HBV infection, viral DNA persists in the liver - though its replication is suppressed by B- and T-cells or by HBV antivirals[3,4]. With immunosuppression, HBV can reactivate, even in patients with past or resolved infection. In untreated patients with chronic or prior HBV, nearly 40% of those receiving chemotherapy for hematological malignancies or solid tumors develop HBV reactivation[5]. HBV reactivation frequently interrupts chemotherapy and increases cancer mortality[6] by causing hepatitis (33%), liver failure (13%) and death (5%)[7]. Use of prophylactic HBV antivirals in patients with chronic or prior HBV infection largely prevents reactivation[1,7-11]. As most patients are asymptomatic and unaware of their HBV infection, hepatitis B serology before immunosuppression is the most effective means to identify the potential for reactivation[12]. Due to a higher HBV prevalence in lymphoma patients than the general population, HBV reactivation is a particular concern in lymphoma[13].

Anti-CD20 antibodies (Ab), such as rituximab, ofatumumab and obinutuzumab, are common treatments for non-Hodgkin's lymphoma (NHL), chronic lymphocytic leukemia (CLL), and rheumatoid arthritis. Anti-CD20 Ab act by depleting B-lymphocytes[14]. However, anti-CD20 Ab also decrease host immune suppression of HBV, potentially leading to viral reactivation - identified by increases in HBV DNA and alanine aminotransferase (ALT)[5]. Among patients with lymphoma and prior HBV infection receiving rituximab, 10%-60% exhibit HBV reactivation at a median of 3 mo after the last rituximab dose[8,15]. Despite 2007-8 guidance from the Centers for Disease Control and the American Association for the Study of Liver Disease[16] recommending HBV screening before immunosuppression, low screening rates persist nationally[4,17].

In 2013, the FDA reported 32 anti-CD20 Ab-related HBV reactivation fatalities occurring up to 12 mo post-therapy, in whom only 3 (9%) received prophylactic HBV antivirals during treatment and follow-up[10]. In 2013, the American Society for Clinical Oncology (ASCO) recommended universal HBV screening prior to anti-CD20 Ab; the 2014 ASCO Quality Oncology Practice Initiative reported nearly 70% HBV screening rates[10]. HBV screening and antiviral treatment decrease reactivation 10-fold and yield cost savings[18]. Additionally, antiviral treatment cost-effectively decreases lymphoma- and liver-related deaths in those with HBV infection[6].

With these improved outcomes with HBV antivirals, 2015 ASCO recommendations prior to anti-CD20 Ab include: (1) hepatitis B surface antigen (HBsAg) and hepatitis B core antibody (HBcAb) screening; (2) treating patients with chronic HBV with entecavir or tenofovir during anti-CD20 Ab and 6-12 mo following; and (3) use of either prophylactic or prompt on-demand HBV antivirals for HBV reactivation (identified by increased HBV DNA or ALT on every 3 mo testing) in those with prior HBV (HBsAg-, HBcAb+)[10]. While ASCO does not specify care of resolved (HBsAg-, HBcAb+, HBsAb+) HBV, prospective controlled lymphoma studies of anti-CD20 Ab report 20% HBV reactivation rates in resolved HBV without use of prophylactic HBV antivirals, resulting in chemotherapy interruptions, hepatitis, and reverse seroconversion (*i.e.*, HBsAg reappears)[1,11]. These events were prevented with prophylactic entecavir treatment[1]. Overall, prophylactic antivirals are associated with lower HBV reactivation, liver failure and death rates compared to on-demand antivirals[8,10].

The VHA is the largest single-system United States health care provider. Compared to the general United States population[2], Veterans exhibit a 2 to 3-fold higher prevalence of chronic HBV infection[19,20]. We aimed to identify all VA patients initiating anti-CD20 Ab (2002-2014) to assess the use of HBV serologic testing, vaccination, antivirals and the rate and timing of hepatitis B-associated complications during treatment.

MATERIALS AND METHODS

Study design and data source

We performed a retrospective cohort study using the VHA Corporate Data Warehouse (CDW), a national repository of VA electronic medical record data[19]. We ascertained patient demographics, inpatient and outpatient visits, laboratory results, procedures (including hepatitis B vaccination), vital signs, pharmacy data, and vital status. VA Vital Status mortality data is highly accurate, exhibiting 98% exact agreement with dates in the National Death Index[21].

Patient population

All patients initiating anti-CD20 Ab (2002-2014) were identified using VHA pharmacy data. Among these, those who were followed to death or for at least 12 mo after completing anti-CD20 Ab were analyzed in this study. The analysis was exempted by the Durham VAMC Institutional Review Board from review as it was performed for VHA quality improvement. Informed consent was not needed as only anonymized patient information was used in this national quality improvement analysis.

Hepatitis B-related variables and their definitions

Pretreatment hepatitis B vaccination at any prior time was obtained from CDW vaccination records. Pretreatment HBV testing was quantified at any preceding time, within the study period, and within 6 mo of anti-CD20 Ab initiation. However, we identified HBV disease categories by serologic testing during the study period only[22]. Most (about 90%) VHA

Table 1 Baseline characteristics and comorbidities and anti-CD20 antibody treatment indication *n* (%)

Baseline characteristics	
Males	18464 (96)
Mean age (range, SE)	66.6 yr (20.3-97.5, 0.0813)
Median, at risk (range)	478 d (365-4083)
Race	
White	14520 (76)
Black or African-American	2460 (12)
Hispanic or Latino	878 (5)
Native Hawaiian or Pacific Islander	171 (1)
American Indian or Alaska Native	148 (1)
Asian	71 (0)
Missing	1056 (5)
Indication for anti-CD20 antibody treatment	
Non-hodgkin's lymphoma	11384 (66.2)
Chronic lymphocytic leukemia	4,110 (23.9)
Rheumatoid arthritis	2,151 (12.5)
Wegener's granulomatosis	174 (1)
Microscopic polyangiitis	54 (0.3)
Baseline comorbidities	
Alcohol abuse	4286 (24.9)
Substance abuse	1485 (8.6)
Hepatitis C	1369 (8)
Cirrhosis	808 (4.7)
Decompensated liver disease	660 (3.8)
Hemodialysis-dependent renal failure	597 (3.5)
HIV	234 (1.4)
Sexually transmitted disease	25 (0.1)
Total number of patients	19304 (100)

HBV assays were qualitative (or categorical), and as normal ranges were not provided in CDW, numerous serology results were indeterminate. We divided the study population into six pretreatment HBV disease categories: definite chronic HBV infection (HBsAg+ for more than 6 mo regardless of HBV DNA), likely chronic HBV (single pretreatment HBsAg+), past HBV (HBsAg-, HBcAb+, HBsAb-)[10], resolved HBV (HBsAg-, HBcAb+, HBsAb+)[1,11], likely prior vaccination (isolated HBsAb+, HBsAg-, HBcAb-), HBV negative (HBsAg-, HBcAb-) or unknown (with no pretreatment HBV serology or those who could not be categorized). Reverse seroconversion was defined as the reappearance of HBsAg or HBeAg in patients with past or resolved HBV[10]. As earlier reported, the "high-risk period" was defined as the period of anti-CD20 Ab treatment and 12 mo follow-up[15]. In patients negative for HBV (HBsAg-, HBcAb-) before treatment, acute HBV was defined by the appearance of HBsAg+ in the high-risk period. Patients with acute, chronic, past or resolved HBV were categorized as HBV positive, while HBV negative or likely vaccinated patients were categorized as HBV negative.

During the high-risk period, HBV antiviral use (adefovir, entecavir, lamivudine, tenofovir, and telbivudine) was identified using the pharmacy data (yes *vs* no). HBV antiviral treatment was termed "prophylactic" when administered within 3 mo of anti-CD20 Ab initiation and "on demand" following this period. Due to very limited quantitative HBV DNA and HBeAg data, we were unable to identify HBV reactivation by published definitions[4,5,8,15]. The rates and timing of health outcomes in the high-risk period included hepatitis events, liver failure and death (overall, cancer-, liver-, or HBV-related). Outcomes were compared among the pretreatment HBV disease categories and by HBV antiviral use. Hepatitis events were defined as ALT > 2 × baseline (ALT immediately preceding anti-CD20 Ab) and ALT > 2 × upper limit normal (ULN) in the high-risk period[8], while liver failure was defined as hepatitis and an INR $\geqslant$ 1.5[23]. Information on death and cause of death in the high-risk period was retrieved from 2014 vital status information. Hepatitis B-associated death met the liver failure definition and had no other apparent cause of death. Liver-related death was identified by International Classification of Diseases, 9th Edition (ICD-9) prior to death[24], as was NHL/CLL cancer related death (ICD-9 codes 200, 202, and 204.12).

Other study variables

Age, gender, race, baseline comorbidities, and the anti-CD20 Ab indication were ascertained at the time of anti-CD20 Ab initiation. Baseline comorbidities were determined using ICD-9 codes related to cirrhosis, decompensated liver disease, hemodialysis-dependent renal failure, human immunodeficiency virus (HIV), sexually transmitted disease, and alcohol and substance abuse.

Statistical analysis

A biomedical statistician performed the statistical analyses and completed pre-submission statistical review. Baseline patient characteristics were tabulated. Statistical analyses were performed using Stata MP-64 version 13.1 (StataCorp LP, College Station, Texas), and differences were considered statistically significant when the *P*-value was less than 0.05. Cumulative hepatitis, liver failure, and death after anti-CD20 Ab initiation were compared by HBV disease categories (6 pretreatment HBV disease categories plus acute HBV: chronic, past, resolved, acute, negative, vaccinated, and unknown) and differences compared using the χ^2 test. Mean time to hepatitis peak ALT, liver failure, and death relative to anti-CD20 Ab administration and follow-up were also compared by HBV disease group.

RESULTS

Demographics

We identified 19304 patients who received anti-CD20 Ab in the VA from 2002-2014, of whom 14887 had at least 12 mo follow-up after anti-CD20 Ab. Most patients were older white males receiving anti-CD20 Ab with NHL (66%), CLL (24%), or rheumatoid arthritis (12%) (Table 1). Comorbid illnesses included alcohol or substance abuse, hepatitis C, cirrhosis, decompensated liver disease or hemodialysis-

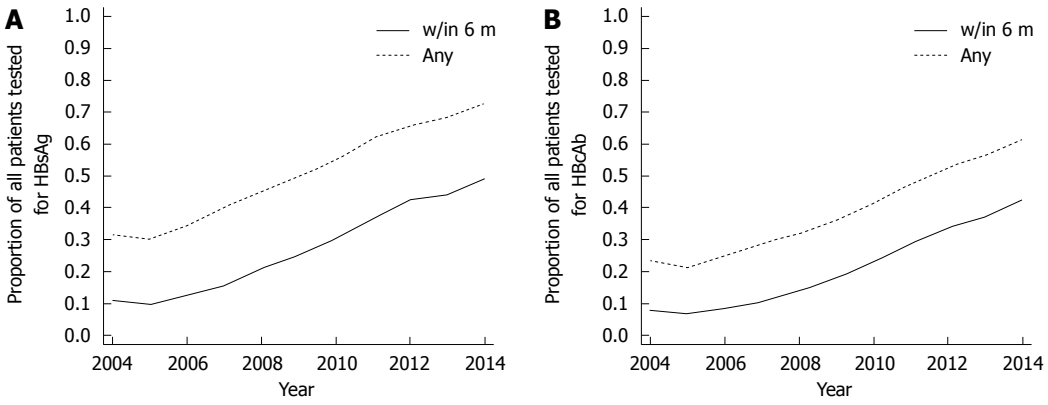

Figure 1 Proportion of all patients with pretreatment hepatitis B surface antigen (A) and hepatitis B core antibody (B) testing over time. Over the study period, pretreatment HBsAg testing within six months of anti-CD20 Ab initiation increased steadily, in parallel with pretreatment HBsAg testing obtained at any time. Over the study period, pretreatment HBcAb testing within six months of anti-CD20 Ab initiation steadily increased, in parallel with pretreatment HBcAb testing obtained at any prior time. HBsAg: Hepatitis B surface antigen; HBcAb: Hepatitis B core antibody.

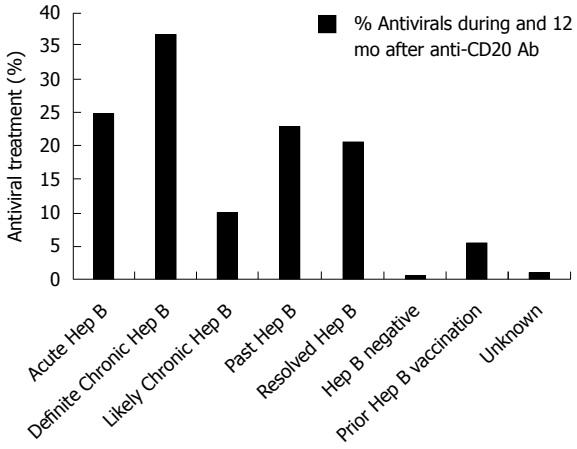

Figure 2 Hepatitis B antiviral treatment by hepatitis B category. Mean hepatitis B antiviral treatment use during anti-CD20 Ab treatment and 12 mo follow-up is profiled by hepatitis B category throughout the study period.

dependent renal failure (Table 1).

Hepatitis B testing

Prior to anti-CD20 Ab treatment, 61%-73% had HBsAg and HBcAb tested at any time pretreatment in 2014 (Figure 1). During the study period, the rates of HBsAg and HBcAb testing increased more than two-fold (Figure 1) with overall pretreatment HBsAg and HBcAb measured in 53% (10224/19304) and 41% (7903/19304), respectively. In 2014, 43%-49% of patients had pretreatment HBsAg and HbcAb screening within 6 mo of anti-CD20 Ab initiation (Figure 1). During the high-risk period for reactivation, < 2% (261/14880) had HBV DNA measured.

Hepatitis B disease categories

In those tested, hepatitis B disease categories (7 categories including "unknown") were assessed as: definite chronic HBV in 40/10224 (0.4%), likely chronic HBV in 127/10224 (1.2%), past HBV in 326/7903 (4%), resolved HBV in 427/8110 (5%), HBV negative in 6002/7903 (76%), likely prior HBV vaccination in 628/8110 (7%), and acute HBV in 0.3% (16/4947) appearing during or after anti-CD20 Ab treatment. The remaining 11723/19304 (61% of overall) patients were termed "unknown," as missing serology or not otherwise categorized. Pretreatment HBV DNA was tested in 2% (403/19304) of patients, of whom 2% (9/403) were positive and 24% (97/403) were indeterminate. At pretreatment baseline, HBeAg was tested in 2% (474/19304), of whom 3% (12/474) were positive and 29% (139/474) were indeterminate. In all HBV categories, 17% or fewer received pretreatment HBV vaccination (as determined by vaccination records).

Antiviral treatment during high-risk period for HBV reactivation

Across all HBV disease categories, few patients receiving HBV antiviral treatment in the high-risk period had concomitant HIV infection (ranging from 1 in 59 to 2 in 9, or 2% to 22%). Overall HBV antiviral use throughout the high risk period ranged from 10%-37% in HBV positive patients at risk for reactivation (Figure 2); the highest rate of HBV antiviral use was 37% in those with definite chronic HBV. In the high-risk period, HBV positive patients exhibited low and variable rates of HBV antiviral treatment throughout the study period (data not shown), although most (80%) HBV antivirals were administered prophylactically (*i.e.*, started within 3 mo of anti-CD20 Ab initiation).

Acute hepatitis B

Among 16 pretreatment HBV negative patients acquiring acute HBV during the high-risk period, the mean peak ALT and bilirubin were 10 × ULN (+/- 13 × ULN) and 7 × ULN (+/- 10 × ULN), respectively (Figure 3). In the 25% (3/12) with acute HBV receiving HBV antivirals, the mean peak ALT was 19 × ULN (*vs* 7 × ULN in those not receiving antivirals). Those with

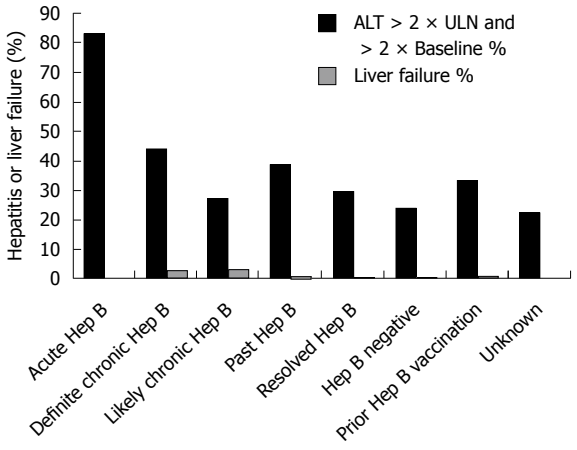

Figure 3 Incidence of hepatitis and liver failure by hepatitis B category. The incidence of hepatitis and liver failure during anti-CD20 Ab treatment and 12 mo follow-up is profiled by hepatitis B category throughout the study period.

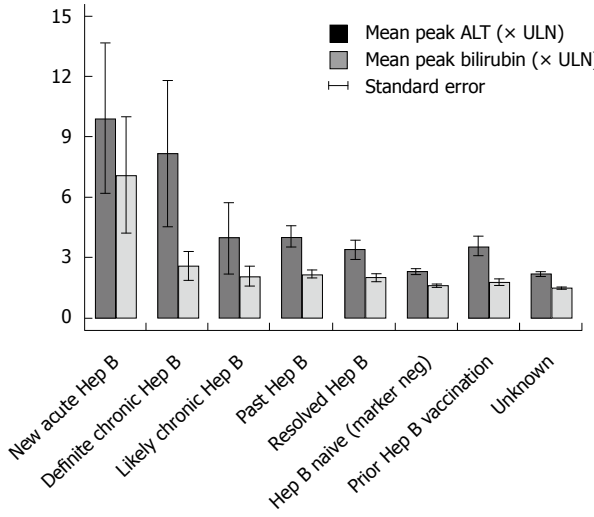

Figure 4 Mean peak ALT and bilirubin by hepatitis B category. The mean peak ALT and bilirubin of patients during anti-CD20 Ab treatment and 12 mo follow-up is profiled by hepatitis B category throughout the study period.

acute HBV exhibited the highest rates of hepatitis [83%) 10/12] among all HBV positive patients, and experienced a 33% (4/12) all-cause mortality (Figure 4). Patients with acute HBV exhibited hepatitis and death at a mean time of 327 d or more following anti-CD20 Ab initiation.

Chronic hepatitis B

During the high-risk period, 37% (11/30) patients with definite chronic HBV received HBV antivirals and exhibited a mean peak ALT and bilirubin of 8 × ULN (+/- 19 × ULN) and 3 × ULN (+/- 4 × ULN), respectively. In contrast, 10% (9/88) with likely chronic HBV received antivirals and had a mean peak ALT 4 × ULN (+/- 16 × ULN) (Figure 3). Among chronic HBV positive patients, those with definite chronic HBV exhibited the highest rates of hepatitis [(43%) 13/30], liver failure [(3%) 1/30] and all-cause mortality [(40%) 12/30], while those with likely chronic HBV exhibited lower rates of

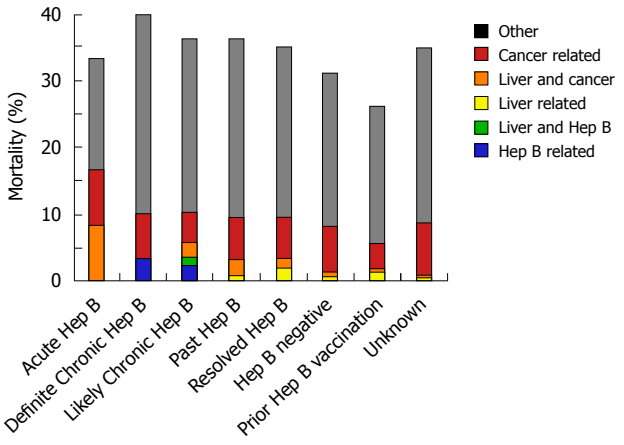

Figure 5 Incidence of overall, hepatitis-B associated, liver-related or cancer-related mortality by hepatitis B category. The overall, hepatitis-B associated, liver-related or cancer-related mortality incidence during anti-CD20 Ab treatment and 12 mo follow-up is profiled by hepatitis B category throughout the study period.

hepatitis [(27%) 24/88], liver failure [(3%) 3/88] and overall mortality [(35%) 31/88] (Figures 4 and 5). Patients with chronic HBV experienced hepatitis and death at a mean time of more than 210 d after anti-CD20 Ab initiation.

Past and resolved HBV infection

Of patients with past and resolved HBV infection, 23% (59/256) and 21% (64/311) received HBV antivirals, respectively, and exhibited a mean peak ALT 3-4 × ULN (+/- 8 × ULN) (Figure 3). Among these patients, those with past HBV exhibited higher rates of hepatitis [(39%) 99/256], liver failure [(1%) 3/256] and overall mortality [(36%) 93/256], while those with resolved HBV exhibited lower rates of hepatitis [(30%) 93/311], liver failure [(0.6%) 2/311] and all-cause mortality [(35%) 109/311] (Figures 4 and 5). Patients with past or resolved HBV developed hepatitis and death at a mean time of 278 d or more following anti-CD20 Ab initiation.

HBV negative patients

In HBV negative patients, 7% (422/6022) had received prior HBV vaccination. HBV antiviral use was associated with concomitant HIV infection. HBV negative patients experienced a mean peak ALT of 2 × ULN (+/-8 × ULN) and the lowest rates of hepatitis [(24%) 994/4143] and liver failure [(0.6%) 27/4143] (Figure 4). These patients had a relatively low all-cause mortality [(31%) 1292/4143] (Figure 5).

Patients likely vaccinated against HBV infection

Patients likely vaccinated against hepatitis B (with isolated HBsAb+) exhibited 2-6-fold higher rates of baseline liver-related comorbidities (24% hepatitis C, 11% cirrhosis and 8% decompensated liver disease), relative to those of unknown HBV status (data not shown). During the high risk period, they had a mean

peak ALT of 4 × ULN (+/-10 × ULN), low rates of hepatitis [(34%) 140/416], liver failure [(1%) 5/416], and the lowest overall mortality [(26%) 109/416] (Figures 4 and 5).

Unknown HBV status

Patients with unknown HBV infection status (as serology missing or incomplete) exhibited the lowest rates of baseline comorbidity and pretreatment HBV vaccination rates [(2%) 252/11718], a mean peak ALT of 2 × ULN (+/-8 × ULN), low rates of hepatitis [(22%) 2163/9631], liver failure [(0.6%) 53/9631], and a moderate overall mortality of [(35%) 3363/9631] (Figures 4 and 5).

Hepatitis significantly higher in HBV positive patients

Patients with acute, chronic, past or resolved HBV infection were categorized as HBV positive, and are at risk of HBV reactivation due to the persistence of HBV DNA. When compared to HBV negative or likely vaccinated patients, the HBV positive patients exhibited significantly higher rates of hepatitis ($\chi^2 = 27.8$, $P = 0.001$), and nonsignificantly higher rates of liver failure and overall mortality. The small numbers of patients on HBV antiviral treatment precluded a planned analysis of health outcomes by HBV disease category in the presence or absence of antiviral treatment.

Relationship between hepatitis B vaccination and overall mortality

Patients with likely prior hepatitis B vaccination (isolated HBsAb+ pretreatment) had the lowest overall mortality rates (26% [108/416]) (Figure 5). In contrast, pre-treatment HBV negative patients who developed acute HBV during the high-risk period experienced a 33% (4/12) mortality rate.

DISCUSSION

In this first 12 year retrospective national VHA analysis, we evaluated HBV testing, vaccination, treatment and outcomes in nearly 20000 Veterans receiving anti-CD20 Ab treatment, largely for NHL or CLL. Rates of pretreatment HBV screening within 6 mo of anti-CD20 Ab initiation more than doubled over the study period. By 2014, most Veterans receiving anti-CD20 Ab had recent pretreatment HBsAg and HBcAb testing and the large majority had testing at any time, which compares favorably with the rates reported in ASCO quality oncology practices[10]. However, few patients susceptible to reactivation had HBV DNA testing during anti-CD20 Ab treatment and follow-up, limiting detection of HBV reactivation. Among those with pretreatment HBV testing, 1 in 9 were HBV positive and at risk for HBV reactivation - yet, only 21% received HBV antivirals during anti-CD20 Ab treatment and follow-up. As a result, HBV positive patients experienced a significantly higher rate of hepatitis than those HBV negative - with most events occurring within one year of treatment initiation. This data aligns with published data reporting the high risk period during anti-CD20 Ab treatment and 12 mo follow-up[15]. These hepatitis events, as well as related morbidity and costs, can be largely prevented with the use of safe, effective prophylactic antivirals in all HBV positive patients throughout the high-risk period of anti-CD20 Ab treatment and 12 mo follow-up[1,7,8,11].

Unexpectedly, we identified 16 cases of acute hepatitis B in the high-risk period arising in patients negative for HBsAg and HBcAb prior to anti-CD20 Ab initiation. These appear to be the first published reports of acute HBV arising *de novo* during anti-CD20 Ab therapy - likely as a result of the prolonged B cell suppression compromising host immune defense[3]. Hepatitis B vaccination substantively decreases the risk of acute HBV, even in high-risk adults[25,26]. Yet, in the current study, only 2% of the nearly 12000 "at risk" HBV unknown and 7% of the 6000 HBV negative patients had pretreatment hepatitis B vaccination. These rates are comparable to the 6% and 9% HBV vaccine immunity rates in Veterans and United States adults age 50 or older, respectively[2].

The strengths of this analysis include its large size, national scope, reliable pharmacy data, relatively high rate of HBV testing, identification of acute HBV risk, diverse indications for anti-CD20 therapy, and 12 mo follow-up of the large majority of patients after anti-CD20 Ab administration. Study limitations include the lack of VHA standardization of HBV serology resulting in some indeterminate results, and the predominantly qualitative HBV serologies. While HBV reactivation is generally identified by logarithmic increases in HBV DNA, reverse seroconversion (newly appearing HBeAg or HBsAg), or increases in ALT[27], the very limited quantified HBV DNA and HBeAg data required us to focus our evaluation on hepatitis - which occurs less frequently than HBV DNA increases in reactivation[4]. Additionally, the effect of HBV antivirals on health outcomes was limited by low antiviral treatment rates.

Automated clinical reminders and decision support have earlier been demonstrated to increase HBV screening and antiviral prophylaxis prior to immuno-suppressive therapy. For example, to increase HBV screening and antiviral prophylaxis in a Spanish medical center, computerized physician order entry prompts for HBV screening when ordering biologic therapies yielded > 90% screening rates, while appropriate consultation and prophylactic HBV antiviral treatment prevented HBV reactivation[28]. As computerized recommendations and follow-on treatment algorithms are highly effective in influencing physician behavior and prescribing[29], computerized decision support may decrease HBV-related disease with anti-CD20 Ab treatment in the VHA.

In conclusion, the VHA now screens most patients for HBV before anti-CD20 Ab treatment, yet seldom

measures HBV DNA during treatment and therefore, likely under-diagnoses HBV reactivation. Increasing VHA hepatitis B vaccination rates should diminish the risk of acute hepatitis B[26] and its complications during anti-CD20 Ab treatment and followup. In HBV positive patients, universal use of HBV antiviral treatment throughout anti-CD20 Ab treatment and 12 mo follow-up will likely decrease mortality and enhance quality of life.

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COMMENTS

Background

Among patients with lymphoma and prior hepatitis B virus (HBV) infection receiving rituximab, 10%-60% exhibit HBV reactivation at a median of 3 mo after the last rituximab dose. Pre-rituximab HBV testing and anti-viral treatment reduces HBV reactivation 10-fold and decreases lymphoma- and liver-related deaths in those with prior HBV infection.

Research frontiers

While the American Society for Clinical Oncology guidelines recommend HBV testing and treatment of patients with prior hepatitis B infection during and up to 12 mo following anti-CD20 antibody therapy, it is unclear how commonly these guidelines are followed in the United States Veterans Health Administration.

Innovations and breakthroughs

This 12 year retrospective cohort study analyzed 19304 Veterans in the United States Veterans Health Administration receiving anti-CD20 antibody therapy. The authors found that pre-treatment HBV testing increased over the study period, yet 37% or fewer received HBV antiviral treatment during anti-CD20 antibody treatment and 12 mo follow-up.

Applications

Results of this analysis can be shared with providers and used to develop electronic health record algorithms to enhance HBV testing and antiviral treatment with anti-CD20 antibody therapy and followup.

Peer-review

This retrospective study presented by Hunt *et al* demonstrates the necessity to screen patients for HBV before anti-CD20 Ab treatment, and most likely, prior to the administration of any immunosuppressive treatment; in order to determine if the patient will benefit from HBV vaccination or preventive antiviral treatment. This simple measure will reduce the number of HBV-related deaths occurring in a number of patients. It is an interesting and relevant study.

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ORIGINAL ARTICLE

Retrospective Study

Contrast-enhanced ultrasound of histologically proven hepatic epithelioid hemangioendothelioma

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Author contributions: Dietrich CF is accepting full responsibility for the conduct of the study, he and all others had access to the data, all had control of the decision to publish; Christoph F planned the study; Dietrich CF, Dong Y, D'Onofrio M, and Cantisani V drafted the manuscript; Dietrich CF and Dong Y performed the statistical analysis; each author made a statement that they approved the final draft submitted; all authors conducted the study, collected and/or interpreted data, and critically revised the manuscript for important intellectual content.

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Abstract

AIM: To analyze contrast-enhanced ultrasound (CEUS) features of histologically proven hepatic epithelioid hemangioendothelioma (HEHE) in comparison to other multilocular benign focal liver lesions (FLL).

METHODS: Twenty-five patients with histologically proven HEHE and 45 patients with histologically proven multilocular benign FLL were retrospectively reviewed. Four radiologists assessed the CEUS enhancement pattern in consensus.

RESULTS: HEHE manifested as a single (n = 3) or multinodular (n = 22) FLL. On CEUS, HEHE showed rim-like (18/25, 72%) or heterogeneous hyperenhancement (7/25, 28%) in the arterial phase and hypoenhancement (25/25, 100%) in the portal venous and late phases (PVLP), a sign of malignancy. Eighteen patients showed central unenhanced areas (18/25, 72%); in seven patients (7/25, 28%), more lesions were detected in the PVLP. In contrast, all patients with hemangioma and focal nodular hyperplasia showed hyperenhancement as the most distinctive feature (P < 0.01).

CONCLUSION: CEUS allows for characterization of unequivocal FLL. By analyzing the hypoenhancement in the PVLP, CEUS can determine the malignant nature of HEHE.

Key words: Guidelines; Recommendations; Liver tumor; Biopsy; Liver transplantation Contrast enhanced ultrasound

Core tip: In this retrospective study, a large cohort of very rare histologically proven hemangioendothelioma (HEHE) was evaluated. Contrast-enhanced ultrasound (CEUS) allowed for improved detection of multilocular HEHE. HEHE showed typical enhancement patterns on CEUS. Therefore, CEUS can help to determine the malignant nature of HEHE.

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INTRODUCTION

Hepatic epithelioid hemangioendothelioma (HEHE) is a rare vascular neoplasm of endothelial origin with primary liver involvement and is characterized by the presence of epithelioid endothelial cells[1]. Weiss and Enzinger first reported 41 patients with this unique tumor in 1982[2]. This tumor is histologically characterized by an epithelial appearance and the endothelial nature of the tumor cells[3]. Currently no more than 200 patients with HEHE have been reported since its first description, and most of the studies were small series[4,5].

No definite etiopathogenetic factors, apart from an association with oral contraceptives, trauma, and exposure to vinyl chloride, have to date been ascribed to HEHE[1,6]. The tumor generally affects adults, with a strong female predominance and a peak incidence occurring between 30 and 40 years of age. The clinical manifestations and laboratory data of HEHE are nonspecific, usually presenting with general symptoms, such as right upper quadrant pain or weight loss. Some patients may present with liver failure, Budd-Chiari syndrome, or portal hypertension, while others may be asymptomatic. Its clinical course and prognosis are variable and unpredictable[7]. Due to its nonspecific clinical manifestations and prolonged clinical course, establishing diagnosis even with histopathological findings can often be challenging[8].

HEHE carries intermediate malignant potential, and transplantation may provide a long term cure[9]. Therefore, the recognition of the imaging features of this rare neoplasm may be helpful for the detection and further surgical treatment of this potentially curable disease. In addition, it is important to distinguish HEHE from other primary and secondary benign and malignant hepatic tumors, such as atypical (multilobulated) hemangioma and hemangiomatosis, hepatocellular adenoma and hepatocellular carcinoma, intrahepatic cholangiocarcinomas, lymphoma, and liver metastases[10]. Radiologists should be aware of its imaging findings and raise suspicion in the proper clinical setting[8,11].

Conventional ultrasound is the most commonly used imaging method for real time diagnosis of FLL. However, the most frequent imaging findings of multilocular HEHE are nonspecific[11]. Often, multiple HEHE on conventional ultrasound might be difficult to differentiate from other atypical multilocular FLLs[12,13]. As a result, the final diagnosis of HEHE depends on biopsy and histological findings[14,15].

Contrast enhanced ultrasound (CEUS) allows for the differentiation of most benign and malignant liver tumors in the portal venous and late phases (PVLP). This finding was summarized in the European Federation of Societies for Ultrasound in Medicine and Biology guidelines and recommendations for the use of CEUS in liver[16,17]. Benign FLLs are typically iso- or hyperenhancing in the PVLP; whereas malignant primary and secondary liver tumors almost always show hypoenhancement in the PVLP, since they do not contain the respective specific hepatic vessels. This hypoenhancement in the PVLP is decisive for determining if a lesion should be biopsied[18,19]. In

Table 1 Baseline characteristics of patients included in our study

Characteristic	HEHE (n = 25)	Hemangioma and FNH (n = 45)
Age (yr)		
mean ± SD	46 ± 14	46 ± 14
Range	24-78	23-74
Male/female	8/17	9/36
Number of FLL (single/multiple)	3/22	0/45
Histological results		
hepatic surgery	6	0
core needle biopsy	19	45

HEHE: Hepatic epithelioid haemangioendothelioma; FNH: Focal nodular hyperplasia; FLL: Focal liver lesions.

addition, CEUS findings of HEHE have not been well addressed. Therefore, the aim of our study is to analyze the CEUS features of histologically proven HEHE and to compare these features to those of other multilocular benign FLLs, including hemangiomas and focal nodular hyperplasia (FNH), since they are the most important for differential diagnosis. We assessed the clinical value of CEUS to define the malignant nature of the disease with hypoenhancement in the PVLP. To our best knowledge, this is the first report on the CEUS features of HEHE.

MATERIALS AND METHODS

Patients

Hemangioendothelioma: Between September 2004 and October 2015, 25 patients (eight male, 17 female, mean age 46 ± 14 years; range 24-78 years) were retrospectively analyzed. In this retrospective study, lesions were histologically proven by hepatic surgery (n = 6) or by 18-gauge core needle biopsy (n = 19).

Three patients had a single FLL, whereas 22 patients had multiple FLLs (Table 1). In patients with multiple FLLs, the selected lesions were those in which biopsies had been performed.

Multilocular hemangioma and FNH

Forty-five patients (nine male, 36 female, mean age 46 ± 14 years; range 23-74 years) with multilocular hemangioma and FNH were also retrospectively analyzed. All lesions were histologically proven by 18-gauge core needle biopsy.

Examination technique

Conventional ultrasound and CEUS were performed by five ultrasound systems: LOGIQ E9 (GE Healthcare, Milwaukee, WI, United States; C1-5 convex array probes, 1-5MHz), Acuson Sequoia (Siemens Healthcare, Erlangen, Germany, 3.5 MHz), Philips iU22 unit (Philips Healthcare, Bothell, WA, United States; C5-1 convex array probes, 1-5MHz), Technos MPX Scanner, and MyLab70 (Esaote, Genova, Italy; ca431 convex array probe 1-8 MHz).

CEUS was performed using contrast harmonic real time imaging at a low MI 0.05-0.30. Each examination lasted about 5 min after the bolus injection. The contrast agent used was SonoVue® (Bracco Imaging Spa, Milan, Italy). For each CEUS examination, a dose of 1.5-2.4 mL of SonoVue® was injected as a quick bolus *via* a 20 gauge intravenous catheter placed in the cubital vein, followed by 5-10 mL of 0.9% normal saline flush. Repeated injection of SonoVue® was performed when necessary.

To characterize the lesion, SonoVue® enhancement during the arterial phase (10-30 s), portal venous (20-120 s), and late vascular phases (120-300 s) were evaluated[17]. All examinations were digitally recorded.

Image analysis

All HEHE images were read by four independent radiologists (15, 17, 23, and 27 years of experience with abdominal ultrasound imaging) blinded to clinical and pathologic data in consensus. Criteria evaluated included number of lesions, maximum diameter, echogenicity (hyperechoic, hypoechoic, or isoechoic; homogeneous or heterogeneous; which were visually compared with the echogenicity of the surrounding liver parenchyma), shape (regular or lobulated), margin (ill- or well defined appearance), and color Doppler imaging features. Using CEUS, the pattern of contrast enhancement of the lesion in comparison to the surrounding liver parenchyma (hypoenhancing, hyperenhancing, isoenhancing), homogeneity of enhancement (homogeneous, heterogeneous), and additional features of enhancement during the arterial, portal venous, and late phases were noted as well, *e.g.,* rim-like or peripheral nodular enhancement, central or eccentric arterial enhancement).

CEUS features of 45 patients of histologically proven multilocular liver hemangioma and FNH were also retrospectively evaluated to compare the CEUS features for differential diagnosis. Digital cineloops were registered both during baseline and post contrast US scanning. All cineloops were digitally stored in a PC based workstation connected to the ultrasound systems.

Pathologic examination

The final pathologic diagnosis was based on hematoxylin-eosin stained sections and immunohistochemical staining results. The immunohistochemical staining included endothelial markers, such as CD 34, CD 31, and factor VIII-related antigen (FVIII Ag)[20].

Statistical analysis

Data are expressed as mean ± SD. All statistical analyses were performed with SPSS 17.0 software package (SPSS, Chicago, IL, United States). The χ^2 test was used to compare HEHE with liver hemangiomas and FNH in terms of enhancement pattern. For the features that played a statistically significant role in

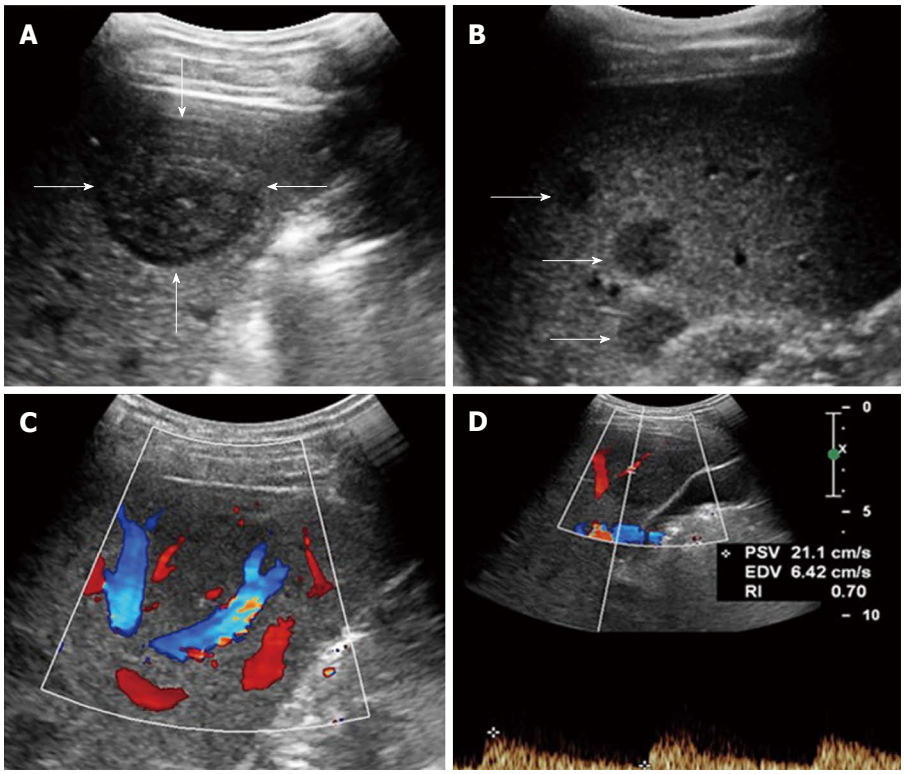

Figure 1 Multiple hepatic epithelioid hemangioendotheliomas in a 31 year female. A: Grayscale ultrasound showed a distinct hypoechoic focal liver lesion (FLL) (arrow); B: Multiple hypoechoic lesions (arrows) were also detected in this patient; C: Color Doppler imaging (CDFI) showed peripheral and intra-lesion color flow signals; D: The resistive index (RI) of color flow was 0.70.

the differentiation diagnosis, we calculated sensitivity and specificity. A difference was considered statistically significant with $P < 0.05$.

RESULTS

Clinical and general pathologic features

All patients were incidentally found to have hepatic lesions by conventional ultrasound screening. Conventional laboratory tests [including transaminases, bilirubin, and gamma-glutamyl transpeptidase (gGT)] were within normal limits or only slightly elevated in all patients. Alpha-fetoprotein, carcinoembryonic antigen, and cancer antigen 19-9 as well as hepatitis B surface antigen and hepatitis C virus were negative respective of normal in all patients.

Final pathologic diagnosis of HEHE showed the typical fibrosclerotic center and cellular periphery on hematoxylin-eosin staining. Immunohistochemically, tumors were positive for at least one endothelial marker, including CD 34 (n = 20), CD 31 (n = 20), or FVIII Ag (n = 11).

Features with conventional ultrasound in HEHE

HEHE manifested as single (3/25, 12%) or multiple FLLs (22/25, 88%) with ill-defined margins on grayscale ultrasound. The lesions were mainly hypoechoic (23/25, 92%) to adjacent liver parenchyma, but a heterogeneous echogenicity with hypo- or hyperechoic FLL was observed (2/25, 8%).

Color Doppler imaging detected branched intralesional vessels in 84% (21/25) of HEHE. The Doppler spectrum was measured in 13 patients. The mean value of resistive index (RI) was 0.64 ± 0.07 (Figure 1 and Table 2).

CEUS features

On CEUS, HEHE presented peripheral rim-like (18/25, 72%) (Figure 2) or heterogeneous hyperenhancement (7/25, 28%) at the arterial phase (Figure 3) and hypoenhancement (100%, 25/25) at PVLP (Figure 4). Central unenhanced areas were observed in 72% (18/25) of HEHE in the late phases. After CEUS, more lesions could be detected in seven patients of HEHE than with conventional ultrasound. Liver hemangioma typically demonstrated peripheral nodular contrast enhancement in all patients, whereas FNH showed central or eccentric arterial blood supply in the arterial phase. In addition, in all patients, both entities showed hyperenhancement in the PVLP, a sign of the benign nature of the lesion. Compared to multilocular liver hemangioma and FNH, characteristic CEUS features of HEHE were peripheral rim-like hyperenhancement in the arterial phase and quick washout in the PVLP with a central unenhanced area in the late phase ($P < 0.01$) (Table 3).

The sensitivity for peripheral rim-like hyperenhancement at the arterial phase was 72%; for

Table 2 Conventional ultrasound features of hepatic epithelioid haemangioendothelioma and hemangioma/Focal nodular hyperplasia *n* (%)

Characteristic	HEHE (*n* = 25)	Hemangioma/FNH (*n* = 45)
Number of nodules (single/multiple)	3/22	0/45
Size of nodules (mm)		
mean ± SD	41.5 ± 25.6	50.4 ± 25.7
range	12-120	20-138
Echogenicity of nodules		
Hyperechoic	2 (8)	19 (42.2)
Hypoechoic	23 (92)	9 (20.0)
Isoechoic	0	17 (37.8)
Homogenous/heterogeneous	9/16	15/30

HEHE: Hepatic epithelioid haemangioendothelioma; FNH: Focal nodular hyperplasia.

quick washout in the PVLP, it was 100%; for central unenhanced area at late phase, it was 72%; and for the combination of both, it was 85% (Table 3).

DISCUSSION

To the best of our knowledge, CEUS features of HEHE have not been well characterized. To date, only a few imaging studies have investigated HEHE, and most of them were limited patients series[4,15,21], and CEUS features of HEHE have been described only in a few patients[15,21,22]. In many patients, CEUS is the first and decisive imaging technique for detecting and characterizing liver tumors[23-25]. The use of ultrasound contrast agents improved detection and made it possible to assess the benign or malignant nature of liver tumors in most patients[13,26-28]. Previously, three forms of HEHE have been described: single nodular, multifocal nodular, and the diffuse type[1]. Consistent with our current study, most HEHE present as multiple FLL. After CEUS, more lesions could be detected in 7/25 (28%) patients[29]. As HEHE has ill-defined margins on grayscale ultrasound, CEUS may be helpful to detect more lesions with sharper and clearer margins.

In our current retrospective study, we discovered that CEUS reliably showed typical signs of HEHE in most patients with hyperenhancement in the arterial phase and hypoenhancement in the PVLP, which might be useful in determining whether a biopsy is necessary for suspected malignant lesions. In correlation with pathologic classification, histologically, HEHE possesses two distinctive characteristics, which are directly related to the echogenicity and enhancement pattern of HEHE on ultrasound images[1,20,30,31]. First, HEHE are composed of dendritic and epithelioid cells with intracytoplasmic lumina containing red blood cells. However, the peripheral proliferation remains active and forms numerous arterial-venous shunts, which

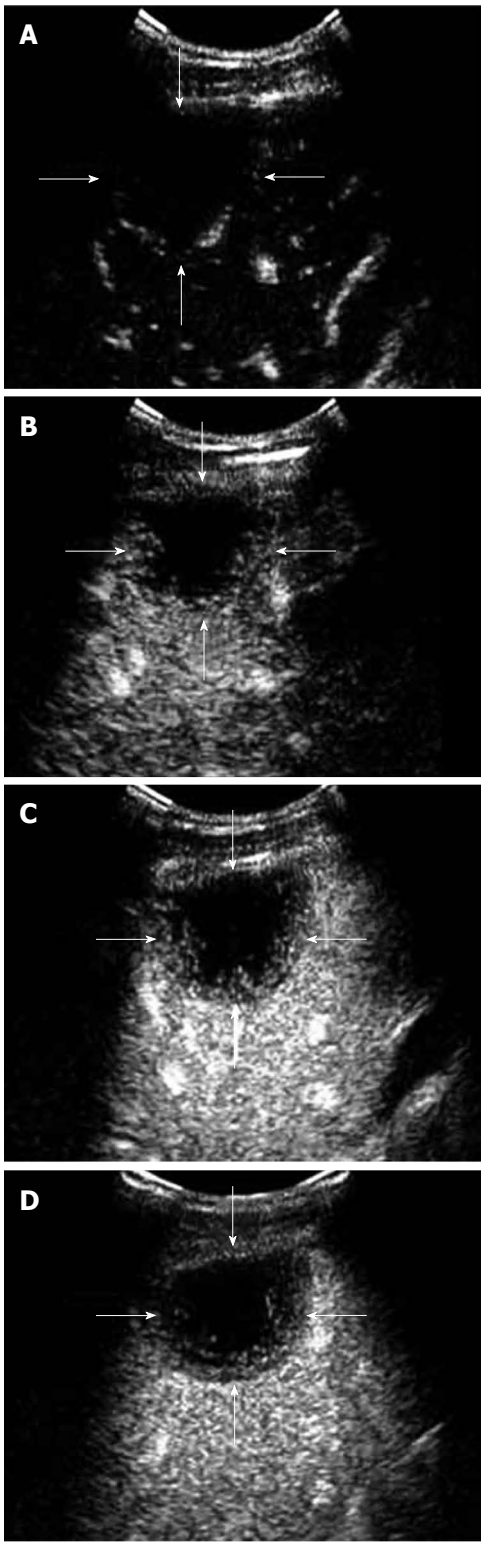

Figure 2 Contrast-enhanced ultrasound feature of hepaticepithelioid hemangioendothelioma in a 31 year female. A: Rim-like enhancement. In arterial phase (16 s after injection of SonoVue), peripheral rim-like enhancement was demonstrated; B: In peak enhancement (24 s after injection of SonoVue), the degree of the rim-like enhancement was equivalent to the liver parenchyma; C: In portal venous phase (45 s after injection of SonoVue), the lesion washed out quickly and showed hypoenhancement; D: In late phase (65 s after injection of SonoVue), the lesion remained hypoenhanced with central unenhanced area.

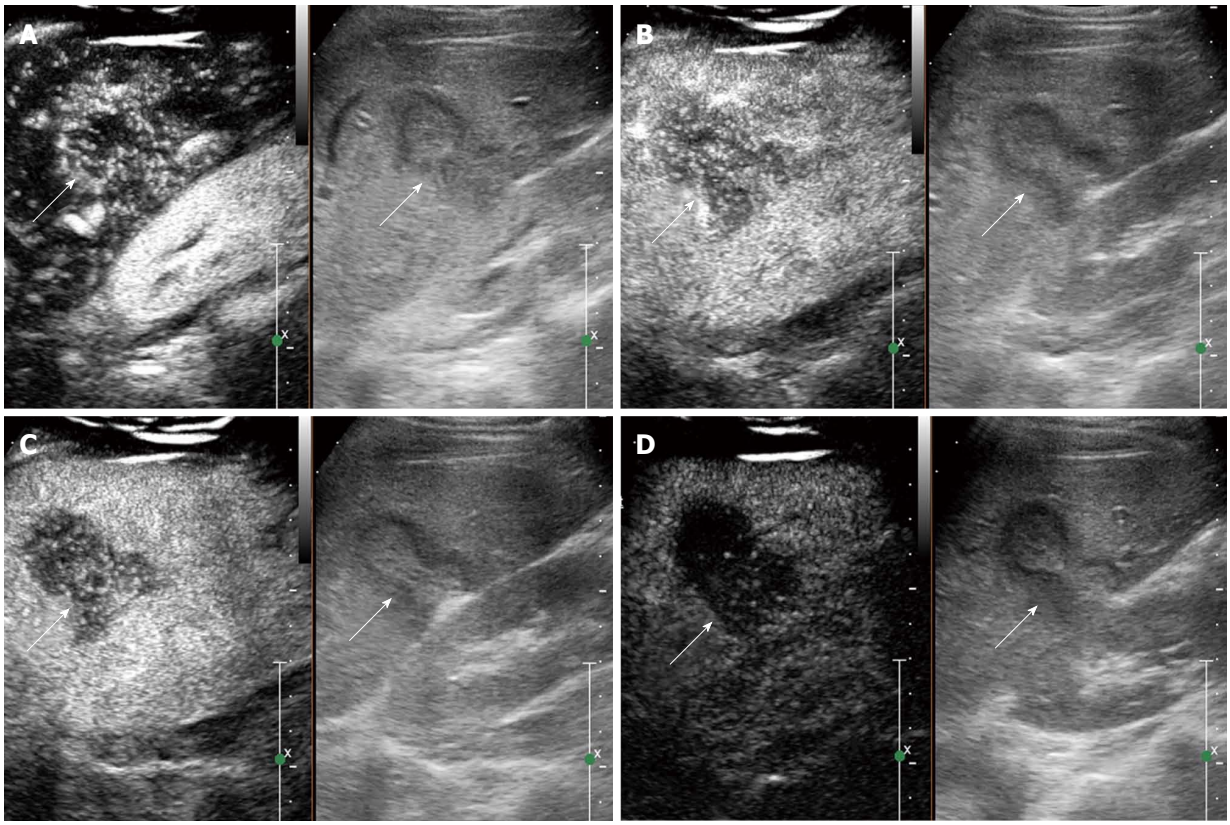

Figure 3 Contrast-enhanced ultrasound feature of hepatic epithelioid hemangioendothelioma in a 25 year female. A: Heterogeneous enhancement pattern. In the arterial phase (16 s after injection of SonoVue), the lesion showed heterogeneous enhancement; B: The enhancement gradually decreased (22 s after injection of SonoVue); C: In the portal venous phase (40 s after injection of SonoVue), the lesion washed out fast than the liver parenchyma and showed hypoenhancement. D: In the late phase (165 s after injection of SonoVue), the lesion remained hypoenhanced.

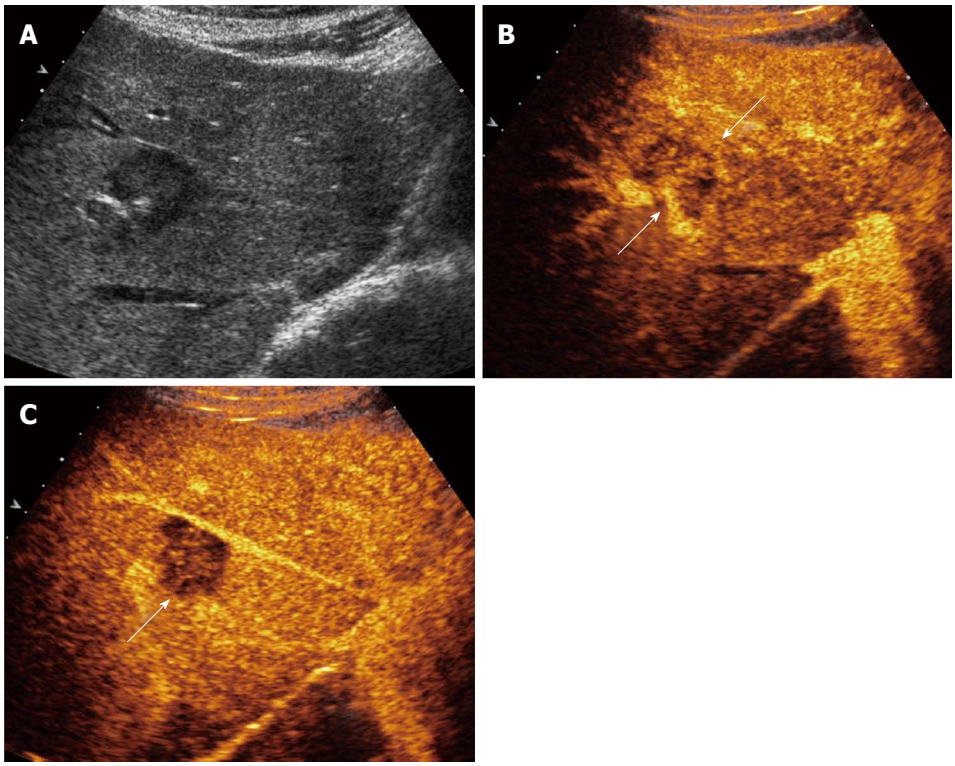

Figure 4 Contrast-enhanced ultrasound feature of hepatic epithelioid hemangioendothelioma in right lobe of liver. A: Grayscale ultrasound showed a hypoechoic focal liver lesions (FLL); B: In the arterial phase the lesion showed heterogeneous enhancement (22 s after injection of SonoVue); C: In the portal venous phase (53 s after injection of SonoVue), the lesion washed out fast and showed hypoenhancement.

Table 3 Contrast enhanced ultrasound imaging features of hepatic epithelioid haemangioendothelioma and multilocular hemangioma/ focal nodular hyperplasia *n* (%)

Characteristic	HEHE (*n* = 25 patients)	Hemangioma/FNH (*n* = 45 patients)
Arterial phase		
Rim like hyperenhancement	18 (72)	0
Heterogeneously hyperenhancement	7 (28)	6 (13.3)
Peripheral nodular enhancement	0	All hemangioma
Central arterial blood supply		All FNH
Portal-venous phase		
Hyperenhancement	0	100 (100.0%)
Hypoenhancement	25 (100)	0
Isoenhancement	0	0
Late phase		
Hyperenhancement	0	45 (100.0%)
Hypoenhancement	25 (100)	0
Isoenhancement	0	0
Sensitivity		
Rim like hyperenhancement	18/25 (72)	0
Hypoenhancement at portal venous phase	25/25 (100)	0
Central unenhanced area at late phase		
Yes	18 (72)	13 (28.9)
No	7 (28)	32 (71.1)

CEUS: Contrast enhanced ultrasound; HEHE: Hepatic epithelioid haemangioendothelioma; FNH: Focal nodular hyperplasia.

could account for the fast rim-like enhancement in the arterial phase and quick washout in the PVLP during CEUS[7]. Second, tumor cells and stroma of HEHE exist in variable proportions, and the central stromal portion of the lesion can vary from myxoid to densely fibrotic. With the growth of the tumor, the central stroma degenerate gradually and become sclerotic as the blood supply decreases[20]. In our results, hypoenhancement with central unenhanced area at PVLP of CEUS was mostly common in HEHE. Moreover, additional lesions were detected at CEUS, leading to improvements in liver staging.

Alomari *et al*[32] first described the lollipop sign as a new cross-sectional sign of HEHE on computed tomography (CT) and magnetic resonance imaging (MRI): a well-defined peripherally enhancing (or non-enhancing) lesion with an avascular core on enhanced images (the candy in the lollipop) and a histologically occluded vein (the stick). Concerning the CT imaging, focal calcifications were reported in 20% of patients; capsular retraction was in 10%-25% of patients[20]. The lesions demonstrated peripheral rim-like hyperenhancement in the arterial phase with even stronger enhancement in the portal venous phase by contrast enhanced MRI. Central areas of reduced signal may correspond to areas of hemorrhage, coagulation necrosis, and calcification[7]. We showed that peripheral rim-like hyperenhancement in the arterial phase and hypoenhancement in the PVLP with central unenhanced areas could be detected in 72% HEHE patients. Therefore, the contrast enhanced image modalities demonstrate a similar enhancement pattern of this disease. CEUS can be considered at least equal to, and in some ways (real time observation, no radiation, less expensive) superior to, CT and MRI as a diagnostic tool[33].

Most of the HEHE lesions were multinodular (88%) and hypoechoic (92%) in our current study. As set out in the current literature and in textbooks, the origin of hypoechoic lesions is considerably more varied and confusing than other lesions[13,23]. All hypoechogenic lesions should be investigated using a contrast enhanced imaging technique[16,18]. Evaluation with CEUS in the PVLP is determinant in this context, and contrast medium hypoenhancement in the late phase is a decisive indication for liver biopsy[23].

HEHE has a variable clinical and biological course compared to benign endothelial tumors (hemangiomas) and malignant angiosarcomas with a slowly progressive phenotype. The tumor can even be difficult to diagnose based on biopsy specimens[34]. CEUS differentiation of different liver tumors is essential because of different therapeutic approaches[35]. HEHE should be differentiated from atypical multilocular liver hemangioma and FNH, because both of them could demonstrate as multilocular hypoechoic liver lesions. Although benign FLLs are commonly iso- or hyperenhancing in the PVLP, malignant primary and secondary liver tumors almost always show hypoenhancement in the PVLP[18,19]. Based on results of our retrospective analysis, we believe that peripheral rim-like hyperenhancement at the arterial phase and quick washout at the PVLP with central unenhanced area are hallmark features that suggest a diagnosis of possible HEHE. In contrast, both multilocular hemangiomas and FNH showed hyperenhancement and remained iso or hyperenhanced in PVLP.

Furthermore, in the clinical setting, factors helpful for the differential diagnosis of HEHE are a medical history without extrahepatic malignant tumor, patients with no symptoms, and laboratory tests[35].

In conclusion, CEUS imaging findings reliably compile typical signs of HEHE, allowing for effective differentiation with other multilocular hypoechoic hepatic lesions, including liver hemangioma and FNH. CEUS can help to improve the diagnostic confidence of HEHE, a rare hepatic tumor, and the liver staging of the disease to guide additional diagnostic work-up.

COMMENTS

Background

To our best knowledge, contrast-enhanced ultrasound (CEUS) features of hepatic epithelioid hemangioendothelioma (HEHE), a rare hepatic tumor, have not been well characterized. To date, only a few imaging studies have investigated HEHE, and most of them were limited patients series.

Research frontiers

This is the first report on the CEUS features of HEHE.

Innovations and breakthroughs

CEUS imaging findings reliably compile typical signs of HEHE and differentiate effectively it from other multilocular hypoechoic hepatic lesions, including liver hemangioma and focal nodular hyperplasia.

Applications

CEUS can help to improve the diagnostic confidence and liver staging of HEHE to guide additional diagnostic work-up.

Terminology

CEUS allows for the differentiation of most benign and malignant liver tumors in the portal venous and late phases.

Peer-review

The aim of this retrospective study was to analyze the CEUS features of histologically proven HEHE in comparison to other multilocular benign focal liver lesions, which might be important differential diagnosis, and to assess the clinical value of CEUS to define the malignant nature of HEHE with hypoenhancement in the portal-venous and late phase.

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ORIGINAL ARTICLE

Retrospective Study

Lymph node dissection in esophageal carcinoma: Minimally invasive esophagectomy *vs* open surgery

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Abstract

AIM: To compare lymph node dissection results of minimally invasive esophagectomy (MIE) and open surgery for esophageal squamous cell carcinoma.

METHODS: We retrospectively reviewed data from patients who underwent MIE or open surgery for esophageal squamous cell carcinoma from January 2011 to September 2014. Number of lymph nodes resected, positive lymph node (pN+) rate, lymph node sampling (LNS) rate and lymph node metastatic (LNM) rate were evaluated.

RESULTS: Among 447 patients included, 123 underwent MIE and 324 underwent open surgery. The number of lymph nodes resected did not significantly differ between the MIE and open surgery groups (21.1 ± 4.3 *vs* 20.4 ± 3.8, respectively, $P = 0.0944$). The pN+ rate of stage T3 esophageal squamous cell carcinoma in the open surgery group was higher than that in the MIE group (16.3% *vs* 11.4%, $P = 0.031$), but no differences was observed for stages T1 and T2 esophageal squamous cell carcinoma. The LNS rate at left para-recurrent laryngeal nerve (RLN) site was significantly higher for open surgery than for MIE (80.2% *vs* 43.9%, $P < 0.001$), but no differences were noted at other sites. The LNM rate at left para-RLN site in the open surgery group was significantly higher than that in the MIE group, regardless of pathologic T stage.

CONCLUSION: For stages T1 and T2 esophageal squamous cell carcinoma, the lymph node dissection result after MIE was comparable to that achieved

by open surgery. However, the efficacy of MIE in lymphadenectomy for stage T3 esophageal squamous cell carcinoma, particularly at left para-RLN site, remains to be improved.

Key words: Esophageal cancer; Lymph node; Minimally invasive; Surgery

Core tip: Previous studies have not reported in detail whether minimally invasive esophagectomy (MIE) can achieve the same lymph node dissection results as open surgery. In particular for esophageal squamous cell carcinoma, it remains unknown whether MIE can meet the technical requirements for each anatomical site in lymph node dissection from the mediastinum to the upper abdomen. Our study found that for stages T1 and T2 esophageal squamous cell carcinoma, the lymph node dissection result after MIE was comparable with that after open surgery. However, the efficacy of MIE in lymphadenectomy for stage T3 esophageal squamous cell carcinoma, particularly at left para-RLN site, remains to be improved.

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INTRODUCTION

Esophageal carcinomas are a group of malignant tumors with poor prognoses. Among esophageal carcinomas, squamous cell carcinoma has a particularly poor prognosis, primarily because of extensive lymph node metastasis in three anatomical regions: the neck, mediastinum, and upper abdomen[1-8]. From 1980s, Japanese surgeons started to investigate three-field lymph node dissection to improve the prognosis of esophageal squamous cell carcinoma. In several studies, the 5-year survival was reported to be improved by approximately 10%. However, three-field lymph node dissection has not been widely accepted because of its complicated procedure and high risk of postoperative complications[9-12].

Minimally invasive esophagectomy (MIE) has been into a rapid development period. Its safety and efficacy to improve patients' life quality have been demonstrated in previous reports[13-18].

However, previous studies have not reported in detail whether MIE can achieve the same lymph node dissection results as open surgery. In particular for esophageal squamous cell carcinoma, it remains unknown whether MIE can meet the technical requirements for each anatomical site in lymph node dissection from the mediastinum to the upper abdomen. This study attempts to retrospectively review the data from patients with esophageal squamous cell carcinoma who were treated at Shanghai Chest Hospital and compare the lymph node dissection results of MIE and open surgery.

MATERIALS AND METHODS

A total of 1343 patients who underwent surgeries to treat esophageal carcinoma at Shanghai Chest Hospital from January 2011 to September 2014 were retrospectively analyzed. The inclusion criteria were as follows: (1) diagnosed with squamous cell carcinoma; (2) underwent either open surgery or MIE; (3) received thoraco-abdominal two-field lymph node dissection; (4) *via* right-side thoracotomy; and (5) the esophageal-gastric anastomosis site was either at the thoracic apex or neck. All the surgeons involved in this study were experienced in both open and thoracoscopic esophagectomy and followed the same principle and technical requirement of lymph node dissection.

To better evaluate the efficacy of the surgeries for dissecting lymph nodes in different anatomical sites under thoracoscopy and laparoscopy, the mediastinal and abdominal lymph node metastasis regions were regrouped for this study (Table 1).

Preoperative evaluation

All patients received enhanced chest and abdominal computed tomography (CT) examinations, cervical CT scan examination or ultrasonic examination, and upper gastrointestinal endoscopic examination before operation. Any tumor involved middle and upper thoracic esophagus was examined by bronchoscopy. Positron emission tomography (PET) or PET-CT was used only in those patients who were willing to pay themselves and with possible distal metastasis. Primary tumor and mediastinal lymph node staging by the endoscopic ultrasound (EUS) examination was performed in all patients, except any patient who had such a narrow esophagus that a gastrointestinal endoscope could not pass through. Cranial magnetic resonance imaging (MRI) and bone emission CT scan were used selectively. The preoperative diagnosis of lymph node metastasis was based on radiology or EUS, endobronchial ultrasound or ultrasound-guided fine-needle aspiration biopsy. Patients who were diagnosed with cT_3 and cN_2 would receive inductive treatment after informed consent was obtained.

Surgical technique

The tri-incisional approach (McKeown) was adopted as the surgical approach for MIE. The esophageal-gastric anastomosis was performed at the neck.

Table 1 Group of lymph nodes

Region	Group
Upper mediastinal region	Right para-recurrent laryngeal nerve (RLN)
	Left para-RLN
	Upper para-esophagus
Carinal and hilar region	Subcarinal
	Left and right para-bronchi
Middle-low para-esophageal region	Middle and low para-esophagus
	Supra-diaphragm
Intraperitoneal region	Para-cardia
	Lesser gastric curvature
	Para-celiac artery and left gastric artery

Patients were in the left lateral recumbent position and leaned forward 30° while the esophagus in the thoracic cavity was freed, and the lymph nodes in the thoracic cavity were dissected under artificial pneumothorax. Afterward, patients were in the supine position while patients' stomachs were freed, and the lymph nodes in the upper abdominen were dissected under laparoscopy. A small midline abdominal incision (8 cm) was made below the xiphoid process to allow completion of the tailoring of the tubular stomach, which was uplifted to the neck to be anastomosed to the esophagus via the substernal or posterior mediastinal pathway. The McKeown or Ivor Lewis approach was used for the open surgery, and the thoraco-abdominal two-field lymph node dissection was required for both approaches.

Evaluation indices included the number of lymph nodes resected, lymph node metastatic (LNM) rate and positive lymph node (pN+) rate in different T stages, and the lymph node sampling (LNS) rate and LNM rate at different sites in the two groups.

Statistical analysis

All statistical analyses were performed with SPSS version 20 (IBM Corp., Armonk, NY). Continuous variables are expressed as mean ± SD. Comparisons of categorical variables were done using χ^2 or Fisher's exact test, and those of continuous variables were done using Student's *t*-test. *P*-values less than 0.05 were considered statistically significant.

RESULTS

A total of 447 patients who met the inclusion criteria were included in this study. Of all the included patients, 324 underwent open surgery (226 males and 98 females with a mean age of 60.3 years), and 123 underwent MIE (97 males and 26 females). There were no significant differences in baseline characteristics, including gender, height, weight, smoking history, the American Society of Anesthesiologists (ASA) score and the rate of complete resection (Table 2). The postoperative pathological diagnostic results revealed

Table 2 Characteristics of the open surgery and minimally invasive esophagectomy groups

	Open surgery (*n* = 324)	MIE (*n* = 123)	*P* value
Age (yr)	60.3 ± 7.8	60.1 ± 6.3	0.782[1]
Gender			0.055[2]
Male	226	97	
Female	98	26	
ASA grade			0.820[2]
I	15	6	
II	248	97	
III	61	20	
Height (cm)	170.2 ± 4.8	171.1 ± 4.9	0.585[1]
Weight (kg)	65.2 ± 9.8	66.8 ± 8.1	0.152[1]
Smoker			0.912[2]
Never	123	46	
Current or former	201	77	
pT stage			
Tis	3	4	0.077
T1	20	36	< 0.001
T2	46	72	< 0.001
T3	225	11	< 0.001
T4	30	0	< 0.001
Resection rate			0.479[2]
R0	313	121	
R1	6	2	
R2	5	0	
Inductive chemo/radio therapy	40%	14%	0.788[2]
pTis/T1/T2	0	0	
pT3	15	6	0.912[2]
pT4	25	0	0.002
LNM rate			
pTis	0	0/4	
pT1	6 (30)	10/36, 27.8%	0.860[2]
pT2	20 (43.4)	29/72, 40.3%	0.731[2]
pT3	125 (55.6)	5/11, 45.5%	0.511[2]
pT4	22 (73.3)	0	
Surgical approach			< 0.001
Macheen	225	123	
Ivor-Lewis	99	0	

[1]The *t* test; [2]Fisher's exact test. LNM rate = number of patients with positive lymph nodes/number of total patients. LNM: Lymph node metastatic; MIE: Minimally invasive esophagectomy; ASA: American Society of Anesthesiologists.

that there were no significant differences in the LNM rates between the open surgery group and MIE group in different T stages. Only 6 patients received inductive chemo/radio-therapy in the MIE group, and those patients' postoperative pathological stages were all T3. Forty patients received inductive chemo/radio-therapy in the open surgery group, of whom 15 were in stage pT3 and 25 in stage pT4. The ratio of the number of patients who received inductive chemo/radio-therapy to the total number of patients in the open surgery group was higher than the ratio in the MIE group; however, there was no significant difference between the two groups.

Table 3 lists the lymph node dissection results for open surgery and MIE in different T stages. The number of lymph node resected did not significantly differ between the MIE and open surgery groups

Table 3 Comparison of the number of lymph node dissections and the rate of positive lymph nodes according to pathological T stage *n* (%)

	Number of lymph node dissections		*P* value[1]	Rate of positive lymph nodes		*P* value[2]	χ^2
	Open	MIE		Open	MIE		
pTis	18.3 ± 1.5	19.8 ± 1.3	0.2150	0	0		
pT1	19.3 ± 4.1	21.4 ± 3.8	0.0593	21 (5.0)	50 (5.8)	0.579	0.308
pT2	20.2 ± 3.2	22.1 ± 6.6	0.0715	98 (10.1)	190 (11.0)	0.494	0.468
pT3	20.3 ± 5.8	23.2 ± 4.1	0.1030	733 (16.3)	32 (11.4)	0.031	4.626
pT4	21.5 ± 3.6	0		130 (17.8)	0		
Total	20.4 ± 3.8	21.1 ± 4.3	0.0944	982 (14.8)	272 (9.5)	< 0.001	49.222

[1]The *t* test; [2]The χ^2 test. The rate of positive lymph nodes (pN+) = number of metastatic lymph nodes/number of removed lymph nodes. MIE: Minimally invasive esophagectomy.

Table 4 Comparison of lymph node sampling rates according to pathological T stage (Including Tis, T1-T4)

		LNS rates		*P* value[1]	χ^2
		Open (*n* = 324)	MIE (*n* = 123)		
Upper mediastinum	R-RLN	262	100	0.916	0.011
	L-RLN	260	54	< 0.001	56.345
	Upper para-esophagus	272	102	0.794	0.068
Subcarinal and parabronchial	Subcarinal	307	112	0.150	2.074
	Left and right parabronchial	310	120	0.353	0.863
Mid and low para-esophagus and diaphragm	Mid and lower para-esophagus	299	110	0.334	0.933
	Diaphragm	128	46	0.683	0.167
Intraperitoneal	Para-cardial	310	116	0.541	0.374
	Lesser gastric curvature	275	105	0.897	0.017
	Left gastric artery	272	106	0.560	0.339

[1]The χ^2 test. LNS rates = number of patients undergoing lymph node sampling/number of total patients. LNS: Lymph node sampling; RLN: Recurrent laryngeal nerve; MIE: Minimally invasive esophagectomy.

(20.4 ± 3.8 *vs* 21.1 ± 4.3, respectively, $P = 0.0944$). There were no significant differences in the pN+ rates between the pT1 stage patients in the two groups or between the pT2 stage patients in the two groups. However, the pN+ rate of the pT3 patients in the open surgery group was significantly higher than the pN+ rate of the patients in the MIE group (16.3% *vs* 11.4%, $P = 0.031$, $\chi^2 = 4.626$). In addition, the overall pN+ rate of the patients in the open surgery group was higher than that in the MIE group (14.8% *vs* 9.5%, $P < 0.001$, $\chi^2 = 49.222$).

Lymph node dissection results in different anatomical regions (the upper mediastinum, left and right para-RLNs, carina and hilus, lower mediastinum, peritoneum, *etc*.) are summarized in Table 4. Comparison of the LNS rates of the two groups revealed that the LNS rate at the left para-RLN site in the patients in the open surgery group was significantly higher than that in the patients of the MIE group (80.2% *vs* 43.9%, $P < 0.001$, $\chi^2 = 56.345$); there were no significant differences in the LNS rates at other sites between the two groups. Table 5 lists the results of the LNM rates of the two groups. The LNM rates in the upper mediastinal region (including the left and right para-RLNs and upper para-esophagus), lesser gastric curvature and middle and lower para-esophagus of the patients in the open surgery group were significantly higher than those in the MIE group. However, the results of stages pTis, T1 and T2 patients indicated that only the LNM rate at the left para-RLN site in patients in the open surgery group was significantly higher than the rate in the MIE group (10.1% *vs* 3.6%, $P = 0.045$); there were no significant differences in the LNM rates at other sites.

DISCUSSION

Esophageal carcinomas rank 7th on the list of fatal tumors and 4th on the list of fatal tumors among male patients. Esophageal squamous cell carcinoma, one type of esophageal carcinoma, is prevalent among Asian populations and has an incidence rate of more than 90%. Different from the conservative approaches that are often adopted in Western countries for treating esophageal carcinomas, radical surgical resection combined with systematic lymph node dissection has always been used as a significant approach for treating esophageal carcinomas in Asian countries, for example in Japan. Although neoadjuvant therapy has been increasingly accepted, surgeries remain the most valuable approach for treating esophageal carcinomas. Lymph node metastasis along the long axis of the esophagus can get to the neck in the upward direction and to the level of the celiac trunk in the downward direction. Given the previous studies, radical tumor

Table 5 Comparison of lymph node metastatic rates according to pathological T stage

		pTis, T1-4		*P* value	χ^2	pTis, T1-2		*P* value	χ^2
		Open (*n* = 324)	MIE (*n* = 123)			Open (*n* = 69)	MIE (*n* = 112)		
Upper mediastinum	R-RLN	71	10	0.001	11.416	6	9	0.876	0.024
	L-RLN	30	4	0.032	4.578	7	4	0.045[2]	
	Upper para-esophagus	40	2	0.001	12.034	1	2	0.999[2]	
Subcarinal and parabronchial	Subcarinal	36	11	0.505	0.445	5	8	0.979	0.001
	Left and right parabronchial	30	11	0.918	0.011	5	9	0.847	0.037
Mid and low para-esophagus and diaphragm	Mid and lower para-esophagus	59	10	0.008	6.939	6	9	0.876	0.024
	Diaphragm	3	1	0.910	0.013	1	1	0.999[1]	
Intraperitoneal	Para-cardial	42	13	0.426	0.634	8	12	0.854	0.034
	Lesser gastric curvature	15	0			3	0	0.042[1]	
	Left gastric artery	51	13	0.163	1.944	8	13	0.998	0.001

[1]The Fisher's exact test; [2]The χ^2 test. LNM rate = number of patients with positive lymph nodes/number of total patients. LNM: Lymph node metastatic; RLN: Recurrent laryngeal nerve; MIE: Minimally invasive esophagectomy.

resection and lymph node dissection (as extensive as possible) may be used to improve the prognosis of a patient with a low tumor load, particularly with a number of metastatic lymph nodes within N2.

The MIE technique has become increasingly popular, and this has been particularly remarkable in China[14]. Currently, there are no universally accepted criteria that determine which patients can receive MIE treatment. Whether MIE can be performed often depends on the experience of the surgeon. Many studies have focused on investigating whether MIE has the same safety level and capabilities for controlling tumors and improving the long-term prognosis and quality of life of patients as open surgery[13,14,16,17,19-23]. An important European randomized controlled trial demonstrated that MIE can better protect the pulmonary function of patients and can improve patients' long-term quality of life[24]. However, the exact oncological surgical results of MIE were not described in detail; the results only showed that the LNS rate of the patients in the MIE group was higher than that of the patients in the open surgery group. However, thorough lymph node dissection is particularly important in treating esophageal squamous cell carcinoma. MIE is affected by such aspects as the position of the patient, the assistant exposing technique and the learning curve; however, many aspects merit more study. To address this issue, a detailed retrospective analysis was conducted in this study. Our research indicates that there were no significant differences in the number of lymph nodes resected and the pathologic LNM rates between the MIE group and the open surgery group in different T stages, demonstrating that MIE can achieve the comparable staging and prediction results with open surgery in terms of lymph node dissection. However, the pN+ rate of the stage T3 patients in the MIE group was significantly lower than that in the open surgery group (11.4% *vs* 16.3%, $P = 0.031$, $\chi^2 = 4.626$); there were no significant differences in the pN+ rates for stages T1 and T2 esophageal squamous cell carcinoma between the two groups. Such a phenomenon has several causes. First, the preoperative patient screening was biased – stages T1 and T2 patients with even lower N stages were more likely to be selected to undergo MIE treatment; thus, MIE could achieve the comparable lymph node dissection results with open surgery for stages T1 and T2 esophageal squamous cell carcinoma. Second, MIE did not reach the same *en bloc* lymph node dissection level as open surgery, therefore, the obtained numbers of positive lymph nodes of patients with advanced stages were relatively low, and hence, it is necessary to provide such high-risk patients with more positive preoperative induction and postoperative adjuvant treatments. Third, the lymph node dissection results of the stage T3 patients in the MIE group were inferior to the results of the open surgery group; however, because only 11 patients were included in the MIE group in this study, it is necessary to increase the sample size to more thoroughly evaluate the difference between MIE and open surgery in terms of the lymph node dissection results of stage T3 patients.

A comparison of lymph node dissection results at specific anatomical sites was also done. The outcome reflected the limitation of the surgical technique used in the patients in the MIE group in this study. The LNS rate at the left para-RLN site (the most difficult site for exposing lymph nodes) of the patients in the MIE group was only 43.9%, whereas this value in the patients in the open surgery group was as high as 80.2%, indicating a significant difference between the two groups. This study further analyzed the LNM rates at different anatomical regions. In terms of the overall LNM rate (including Tis and T1-4), the LNM rates in the upper mediastinal region of the patients in the MIE group were lower than the rates of the patients in the open surgery group. The patients in the MIE group were primarily stages T1 and T2 patients. To

eliminate the effect generated by the biased inclusion process, we analyzed the stages T1 and T2 patients in separate groups. It has been proved that the LNM rate at the left para-RLN site in the MIE group was significantly lower than that in the open surgery group, but there were no significant differences in the other regions. Hence, lymph node dissection at the left para-RLN site remains a key technique of MIE that requires improvement. Currently, the following techniques were adopted to rectify the aforementioned shortcomings: (1) a single-lumen endotracheal tube-aided blocker is used to reduce the tracheal tension to allow easier exposure of the left space of the trachea during surgery; (2) the left RLN is moved upward through the assistant traction of the esophagus to allow easier lymph node dissection anterior to the nerves; and (3) the auxiliary artificial pneumothorax is used to enlarge the mediastinal space. After using these techniques, the LNS rate at the left para-RLN site recently increased to above 90%, which is similar to the results of the previously mentioned study.

Limitations of the study

This study was a single-center retrospective study; during the medical case accumulation process, the initial learning curve may have affected the results. In addition, the sample size is not sufficiently large; in particular, there are few stage T3 patients (only 11 patients) in the MIE group. However, considering the fact that the current MIE technique remains applicable to stages T1 and T2 patients, this study nevertheless reflects the basic surgical oncological results of the current MIE technique. Furthermore, this study did not statistically analyze the patients' survival rates, therefore, the best evidence for the lymph node dissection effect in MIE is lacking. In the future, a multicenter prospective randomized controlled study with a large sample size is expected to be conducted to verify the lymph node dissection effect of MIE.

In summary, we conducted a retrospective comparative study of MIE and conventional open surgery for treating esophageal squamous cell carcinoma. The initial results indicate that MIE could achieve the comparable lymph node dissection results with the open surgery, particularly for stages T1 and T2 esophageal squamous cell carcinoma. However, the lymph node dissection at the left para-RLN site remains a major technical challenge for MIE.

COMMENTS

Background

Previous studies have not reported in detail whether minimally invasive esophagectomy (MIE) can achieve the same lymph node dissection results as open surgery. In particular for esophageal squamous cell carcinoma, it remains unknown whether MIE can meet the technical requirements for each anatomical site in lymph node dissection from the mediastinum to the upper abdomen. This study attempts to retrospectively review the data from patients with esophageal squamous cell carcinoma who were treated at Shanghai Chest Hospital and compare the lymph node dissection results of MIE and open surgery.

Research frontiers

MIE has been into a rapid development period. Its safety and efficacy to improve patients' life quality have been demonstrated in previous reports. The main aim of this study was compare lymph node dissection results of minimally invasive esophagectomy and open surgery for esophageal squamous cell carcinoma.

Innovations and breakthroughs

The authors conducted a retrospective comparative study of MIE and conventional open surgery for treating esophageal squamous cell carcinoma. This study is a large cohort. The initial results indicate that MIE could achieve the comparable lymph node dissection results with the open surgery, particularly for stages T1 and T2 esophageal squamous cell carcinoma.

Applications

This study proved that MIE could achieve the comparable lymph node dissection results with the open surgery, particularly for stages T1 and T2 esophageal squamous cell carcinoma. This is very important for the development of MIE.

Terminology

MIE could achieve the comparable lymph node dissection results with the open surgery, particularly for stages T1 and T2 esophageal squamous cell carcinoma. However, the lymph node dissection at the left para-RLN site remains a major technical challenge for MIE.

Peer-review

Previous studies have not reported in detail whether MIE can achieve the same lymph node dissection results as open surgery. In particular for esophageal squamous cell carcinoma, it remains unknown whether MIE can meet the technical requirements for each anatomical site in lymph node dissection from the mediastinum to the upper abdomen. This study found that for stages T1 and T2 esophageal squamous cell carcinoma, the lymph node dissection result after MIE was comparable with that of open surgery. However, the efficacy of MIE in lymphadenectomy for stage T3 esophageal squamous cell carcinoma, particularly at left para-RLN site, remains to be improved.

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ORIGINAL ARTICLE

Observational Study

Chinese physicians' perceptions of fecal microbiota transplantation

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Institutional review board statement: The survey study was performed by asking physicians to assess their perceptions using questionnaires with no risk to the participants, and no individual physician information was revealed under the condition of anonymity. Thus, the study was exempt from the requirement for ethical approval.

Informed consent statement: The need for informed consent in this study was waived by the Chinese PLA General Hospital Institutional Review Board because the study was a survey of physicians' perceptions using questionnaires; there was no risk to the participants, and no individual physician information was revealed under the condition of anonymity.

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Data sharing statement: No additional data for the study are available.

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Abstract

AIM: To explore Chinese physicians' perceptions towards fecal microbiota transplantation (FMT) and to provide information and an assessment of FMT development in China.

METHODS: A self-administered questionnaire was developed according to the FMT practice guidelines and was distributed to physicians in hospitals *via* Internet Research Electronic Data Capture (REDcap) software and electronic mails to assess their attitudes toward and knowledge of FMT. The questionnaire included a brief introduction of FMT that was followed by 20 questions. The participants were required to respond voluntarily, under the condition of anonymity and without compensation. Except for the fill-in-the-blank questions, all of the other questions were required in the REDcap data collection systems, and the emailed questionnaires were completed based on eligibility.

RESULTS: Up to December 9, 2014, 844 eligible questionnaires were received out of the 980 distributed questionnaires, with a response rate of 86.1%. Among the participants, 87.3% were from tertiary hospitals, and there were 647 (76.7%) gastroenterologists and 197 (23.3%) physicians in other departments (non-gastroenterologists). Gastroenterologists' awareness of FMT prior to the survey was much higher than non-gastroenterologists' (54.3 *vs* 16.5%, $P < 0.001$); however, acceptance of FMT was not statistically different (92.4 *vs* 87.1%, $P = 0.1603$). Major concerns of FMT included the following: acceptability to patients (79.2%), absence of guidelines (56.9%), and administration and ethics (46.5%). On the basis of understanding, the FMT indications preferred by physicians were recurrent *Clostridium difficile* infection (86.7%), inflammatory bowel disease combined with *Clostridium difficile* infection (78.6%), refractory ulcerative colitis (70.9%), ulcerative colitis (65.4%), Crohn's disease (59.4%), chronic constipation (43.7%), irritable bowel syndrome (39.1%), obesity (28.1%) and type 2 diabetes (23.9%). For donor selection, the majority of physicians preferred individuals with a similar gut flora environment to the recipients. 76.6% of physicians chose lower gastrointestinal tract as the administration approach. 69.2% of physicians considered FMT a safe treatment.

CONCLUSION: Chinese physicians have awareness and a high acceptance of FMT, especially gastroenterologists, which provides the grounds and conditions for the development of this novel treatment in China. Physicians' greatest concerns were patient acceptability and absence of guidelines.

Key words: Fecal microbiota transplantation; Chinese physicians; Gastroenterologists; Perception; Survey

Core tip: Perceptions and attitudes toward fecal microbiota transplantation (FMT) by physicians and patients play an important role in determining its acceptability. We investigated Chinese physicians' acceptance levels of FMT, their concerns about FMT, and their perspectives of FMT techniques. The few data about the perceptions of physicians toward FMT are all from Western countries; this is the first study of physicians' perceptions of FMT in an Asian country. Additionally, our study was representative with a large respondent number (844) and a large coverage area of China (22 out of 34 provinces); thus it can provide preliminary information for the development of FMT in China.

Ren RR, Sun G, Yang YS, Peng LH, Wang SF, Shi XH, Zhao JQ, Ban YL, Pan F, Wang XH, Lu W, Ren JL, Song Y, Wang JB, Lu QM, Bai WY, Wu XP, Wang ZK, Zhang XM, Chen Y. Chinese physicians' perceptions of fecal microbiota transplantation. *World J Gastroenterol* 2016; 22(19): 4757-4765 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i19/4757.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i19.4757

INTRODUCTION

Fecal microbiota transplantation (FMT) refers to the instillation of fecal suspension from a healthy person into the gastrointestinal (GI) tract of a patient to cure a certain disease by restoring the construction of intestinal flora. FMT is by no means a new concept. Fecal medicine was recorded 3000 years ago in the "Collection of 52 Prescriptions"[1,2], which was

described as the oldest traditional prescription book in China. Later, during the Eastern Han dynasty in the 2nd century A.D. in China, Zhang Zhongjing described the use of a human fecal suspension by mouth to treat food poisoning in "Jin Gui Yao Lüe" (Synopsis of Golden Chamber)[3]. To our knowledge, this was the first literary record of using human fecal liquid to treat diseases. Then, Ge Hong, Sun Simiao, Li Shizhen, *etc*., described a series of prescriptions using fecal suspensions or dry feces to treat abdominal diseases in their famed traditional Chinese medicine books[4-6]. The first description of FMT in Western countries was in 1958, when four patients with pseudomembranous colitis were cured using fecal enemas[7]. However, FMT did not gain public attention until recently and only after several studies reported that fecal suspension had astounding efficacy for recurrent *Clostridium difficile* infection (RCDI)[8,9]. Since then, FMT, an ancient medicine, has become a hot topic and interest has surged in recent years. Currently, more than 40 reports are available about treating RCDI with FMT, with similarly high reported efficacy. FMT was recommended by the American CDI guidelines in 2013 if there was a third recurrence after a pulsed vancomycin regimen[10]. As FMT may restore the dysbiosis of gut microbiota, it is also proposed in treating other GI diseases and non-GI diseases, which have been considered to be linked to the composition of gut microbiome, with associations described between intestinal flora, immune system, and active metabolites[11], such as in inflammatory bowel diseases (IBD), chronic constipation, type 2 diabetes mellitus, metabolic syndrome, and symptoms of Parkinson's disease[12-15]. However, using fecal suspension to treat diseases other than CDI is still speculative, even for IBD.

The perceptions and attitudes toward FMT held by physicians and patients play an important role in determining its acceptability. A few reports discuss patients' attitudes towards the acceptance of FMT[16,17]. Despite the unappealing nature of stool, 46% of patients with ulcerative colitis were willing to accept FMT as a treatment, and if it was recommended by their physicians, up to 94% of patients with recurrent CDI are ready to accept FMT[16]. One study reported that 97% of patients with RCDI who had undergone FMT once were willing to accept the treatment again, and an equal number of patients (53%) chose FMT as the treatment of first choice[17]. Nevertheless, minimal data exist regarding physicians' perception of this technique[18,19]. The acceptance of FMT in Asian countries remains unknown. Therefore, this survey was designed to evaluate Chinese physicians' perceptions, and especially their acceptance of FMT. We will compare the different views about FMT technology, to provide information and an assessment of the future development of FMT.

MATERIALS AND METHODS

The study was conducted from June 2014 to September 2014. A self-administered questionnaire was developed according to the practice guidelines and other literature on FMT[9,20] and was distributed to physicians *via* Internet Research Electronic Data Capture (REDcap) software[21] and emails. The participants were a convenience sample of physicians working in hospitals and practicing gastroenterology; other specialists, such as those physicians working in endocrinology, pediatrics, general surgery, and neurosurgery, were also included in the study. These physicians were recruited through gastroenterology associations and their subspecialty groups in different provinces.

The questionnaire included a brief introduction of FMT, followed by 20 questions, which were comprised of three sections: demographic information of the interviewees, their attitudes toward FMT, and FMT technique-associated questions (see Supplementary material). The participants were required to respond voluntarily and under the condition of anonymity and without compensation. Except for the fill-in-the-blank questions, all other questions were required in the REDcap system. The email questionnaires were completed according to eligibility.

Statistical analysis

Study data were collected and managed using REDCap tools hosted at the General Hospital of the Chinese PLA. REDCap was used to manage study data and perform the descriptive analysis. The data were also analyzed using Microsoft Excel and JMP 10.0.0 software. Continuous data are presented as the mean ± SD and analyzed by the ANOVA test. Categorical data are presented as percentages and were analyzed by the χ^2 test. Univariate analysis and multivariate logistic regression analysis were employed to identify the impact of various factors on physicians' preferences for FMT. Odds ratios (ORs) and 95% confidence intervals were calculated and a *P*-value less than 0.05 was considered statistically significant.

RESULTS

Characteristics of the respondents

Up until December 9, 2014, 844 eligible questionnaires were received out of the 980 distributed questionnaires, with a response rate of 86.1%. Respondents were selected from six different regions of China, and the study included respondents from most areas of China (22 out of 34 provinces). There were 449 (53.2%) females and 395 (46.8%) males with an average age of 36.1 ± 9.2 years (age range: 19-81 years). The majority of respondents were gastroenterologists (76.7%, 647/844), and most of them were associated

Table 1 Characteristics of the survey respondents *n* (%)

Characteristic	*n* = 844
Age, mean ± SD (range)	36.1 ± 9.2 (19-81)
Gender, male	395 (46.8)
Region	
North West	211 (32.7)
North	152 (23.6)
East	100 (15.5)
North East	83 (12.9)
South West	68 (10.5)
South Central	31 (4.8)
Missing data	199
Education	
College degree	295 (35.0)
Postgraduate degree	341 (40.4)
Doctoral degree	188 (22.3)
Post-doctoral degree	19 (2.3)
Professional title	
Resident physician	291 (34.6)
Attending physician	210 (24.9)
Associated chief physician	198 (23.5)
Chief physician	143 (17.0)
Level of hospital	
Community hospital	15 (1.8)
Secondary hospital	88 (10.4)
Tertiary hospital	737 (87.3)
Profession	
Gastroenterologist	647 (76.7)
General surgeons	49 (5.8)
Endocrinologist	28 (3.3)
Others	120 (14.2)
Working time in gastroenterology (yr)	
< 2	295 (35.3)
3-5	111 (13.3)
6-10	106 (12.7)
10-20	188 (22.5)
> 20	135 (16.2)

Regions were classified according to the common geographical zones in China.

with tertiary hospitals (87.3%, 737/844). More than half of the physicians were qualified postgraduates or above, and almost half of the physicians held senior professional titles and had worked in gastroenterology for more than 6 years (Table 1).

Attitudes toward FMT

Among the physicians, 607 (71.9%) had heard of FMT prior to the survey, but only 45.6% (385/844) had an awareness or understanding of FMT (*i.e.*, "had knowledge of FMT principles and technology"). The primary advertising approach included conferences (60.3%, mainly domestic conferences), professional journals (54.8%) and communication with colleagues (42.1%). Gastroenterologists' prior awareness of FMT was much higher than non-gastroenterologists' (54.3 *vs* 16.5%, $P < 0.001$), they were more interested in FMT training (92.4 *vs* 81.4%, $P < 0.001$), and they showed a more positive attitude to the feasibility (74.5 *vs* 59.3%, $P < 0.001$) and potential (71.5 *vs* 53.9%, $P < 0.001$) of FMT. However, the acceptance of FMT was similarly high among gastroenterologists and non-gastroenterologists (92.4 *vs* 87.1%, $P = 0.1603$).

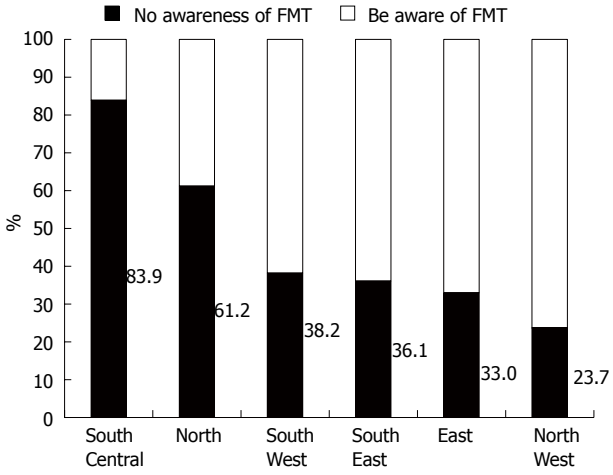

Figure 1 Physicians' awareness of fecal microbiota transplantation in different regions. FMT: Fecal microbiota transplantation.

Table 2 Multivariate analysis of the factors associated with fecal microbiota transplantation awareness

Variable	*P*-vaule	OR	95%CI
Age	0.160	1.391	0.878-2.203
Region	< 0.001[1]		
Region (North)	< 0.001[1]	0.288	0.163-0.508
Region (North East)	0.011[1]	0.385	0.185-0.800
Region (South Central)	0.058	3.005	0.963-9.376
Region (East)	0.089	0.555	0.282-1.093
Region (South West)	0.051	0.467	0.217-1.003
Educational background	< 0.001[1]	1.958	1.402-2.733
Professional title	0.010[1]	1.676	1.133-2.480
Level of hospital	0.069	1.759	0.958-3.228
Department	0.001[1]		
Department (gastroenterology)	< 0.001[1]	4.182	1.895-9.229
Department (general surgery)	0.104	2.429	0.834-7.073
Department (endocrinology)	0.903	0.919	0.235-3.584
Working time on gastroenterology	0.476	1.090	0.860-1.383

[1]$P < 0.05$. Age was divided into 4 groups: ⩽ 30 years, 30-40 years (including 40 years), 40-50 years (including 50 years), > 50 years.

In the univariate analysis, significant factors ($P < 0.05$) that influenced physicians' awareness of FMT included age, educational background, professional designation, level of hospital, region, department and working experience in gastroenterology. The multivariate logistic regression analysis confirmed that physicians with a higher education (OR = 1.958, 95%CI: 1.402-2.733, $P < 0.001$) and a higher professional title (OR = 1.676, 95%CI: 1.133-2.480, $P = 0.010$) were more likely to understand FMT, and gastroenterologists were more likely to comprehend FMT than physicians in other departments (OR = 4.182, 95%CI: 1.895-9.229, $P < 0.001$). Physicians in different regions had significantly different understandings of FMT ($P < 0.001$) (Figure 1 and Table 2).

The acceptance rate of the 385 physicians who had knowledge of FMT was 91.9%. Of these physicians, 59.5% (229/385) were willing to choose FMT ahead of

Table 3 Multivariate analysis of factors associated with fecal microbiota transplantation preference

Variable	*P*-vaule	OR	95%CI
Age	0.672	1.155	0.593-2.250
Region	0.007[1]		
Region (North)	0.838	1.101	0.437-2.773
Region (North East)	0.095	0.412	0.146-1.167
Region (South Central)	0.748	1.419	0.168-11.975
Region (East)	0.096	0.456	0.180-1.151
Region (South West)	0.006[1]	0.264	0.102-0.683
Educational background	0.945	1.016	0.657-1.570
Professional title	0.757	0.913	0.513-1.624
Level of hospital	0.041[1]	0.359	0.134-0.961
Department	0.910		
Department (gastroenterology)	0.510	1.291	0.604-2.760
Department (general surgery)	0.778	1.177	0.379-3.657
Department (endocrinology)	0.598	1.463	0.356-6.020
Working time on gastroenterology	0.683	0.933	0.670-1.299
Understanding of FMT	0.002[1]	3.265	1.555-6.855

[1]$P < 0.05$. FMT: Fecal microbiota transplantation.

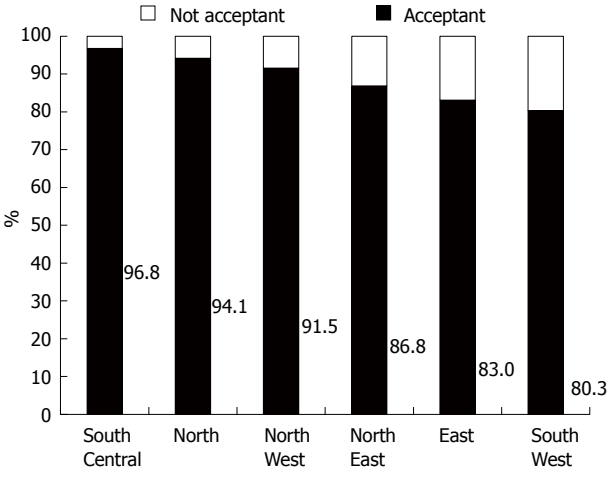

Figure 2 Physicians' acceptance of fecal microbiota transplantation in different regions.

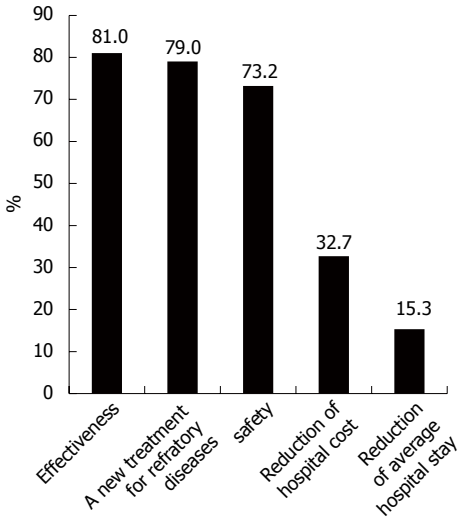

Figure 3 Physicians' concerns about choosing fecal microbiota transplantation as a treatment.

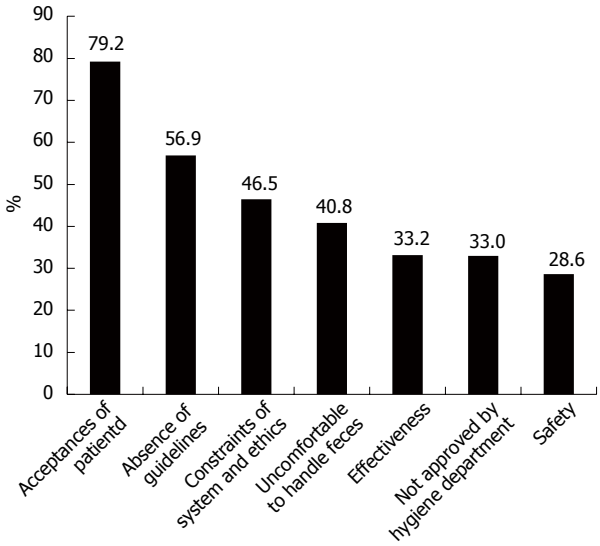

Figure 4 Barriers against clinical applications of fecal microbiota transplantation.

other treatments, and 80.8% (126/156) of physicians who declined FMT as the first treatment selected FMT as an alternative treatment.

A univariate analysis revealed that only geographic region can significantly influence physicians' acceptance ($P < 0.05$). Factoring the significant variables in a univariate analysis and those affecting the acceptance of FMT, such as age, educational background, professional title, hospital level, department, working time in gastroenterology and understandings of FMT into the multivariate logistic regression analysis, it was unexpectedly discovered that understandings of FMT, hospital level and region were all statistically significant (Table 3). Physicians with a greater comprehension of FMT were more likely to accept FMT (OR = 3.265, 95%CI: 1.555-6.855, $P = 0.002$). The higher the level of hospital physicians worked at, the less likely they were to accept FMT (OR = 0.359, 95%CI: 0.134-0.961, $P = 0.041$). The lowest acceptance of FMT (80.3%) was observed among physicians working in Southwest China, followed by those in the East (83.0%). Acceptance rate of physicians was above 85% in all other regions ($P = 0.007$) (Figure 2).

The three most frequent reasons for choosing FMT were as follows: efficacy (81.0%), a new treatment option for refractory diseases (79.0%) and safety (73.2%) (Figure 3). Primary barriers for the clinical application of FMT included patients' acceptance (79.2%), absence of guidelines (56.9%) and systemic and ethical constraints (46.5%) (Figure 4).

Perspectives on FMT technique-associated questions

Although we provided a brief description of FMT in the questionnaire, there were some questions about the details of FMT procedures. Therefore, it might not have been reasonable to ask physicians who had

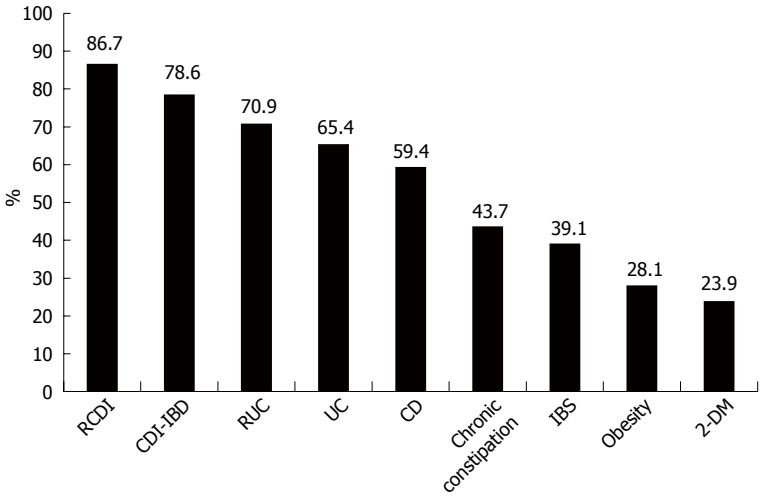

Figure 5 Fecal microbiota transplantation indications. RCDI: Refractory *Clostridium difficile* infection; UC: Ulcerative colitis; CD: Crohn's Disease; RUC: Refractory ulcerative colitis; CDI-IBD: Inflammatory bowel disease with *Clostridium difficile* infection; IBS: Irritable bowel syndrome; 2-DM: Type 2 diabetes mellitus.

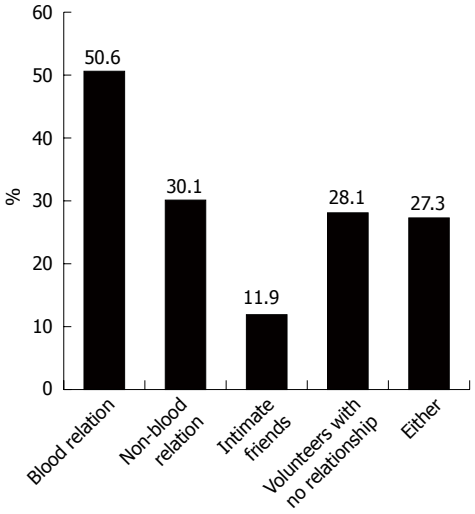

Figure 6 Selection of donors.

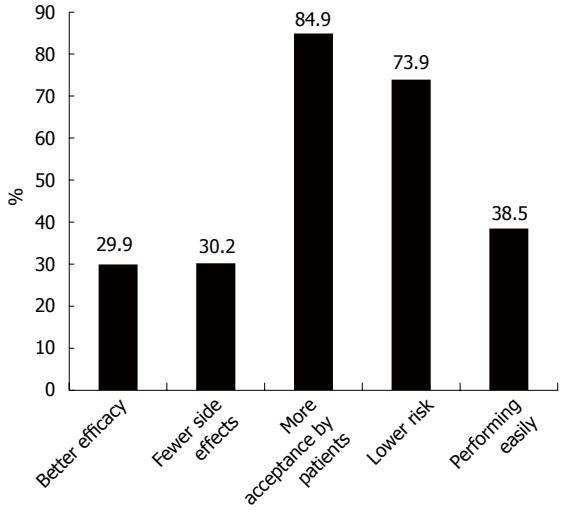

Figure 7 Reasons for lower gastrointestinal tract selection.

no awareness of FMT to analyze FMT technology. To disclose the physicians' true perceptions of FMT procedures, we excluded physicians who had no knowledge of FMT in the following analysis.

Indications: The majority of physicians (86.7%) selected recurrent RCDI, followed by other diseases such as inflammatory bowel disease with CDI, refractory ulcerative colitis, ulcerative colitis, and Crohn's disease (Figure 5).

Donor selection: Most participants preferred someone who had a similar microbiota environment to the recipient, including blood relatives (50.6%), non-blood relatives (30.1%) and intimate friends (11.9%) (Figure 6). Only 28.1% of participants selected volunteers with no relationship, and 27.3% held the view that either of the above was an option contingent on the health of the donor; 29.7% of physicians were more inclined to prefer children donors, 35.4% selected adults, and 34.9% preferred both.

Selection of the administration route: Overall, 76.6% of the respondents preferred the lower GI tract as the route of administration, with the primary reasons being that patients would more likely accept this route (84.9%) and that it had lower risk (73.9%) (Figure 7). Only 13.9% of the physicians selected the upper GI tract, and others (7.1%) thought that both approaches were acceptable. With regard to the site for performing FMT, nearly half of the physicians (44.9%) preferred the Endoscopy Center, and only 21.3% preferred wards.

Risk of FMT: Most participants (69.2%) held the opinion that FMT has a low risk with transient abdominal symptoms such as diarrhea, and 14.4% of physicians thought that FMT had a high and even lethal risk (Figure 8). The vast majority of these respondents thought that disease history (93.5%), stool and blood

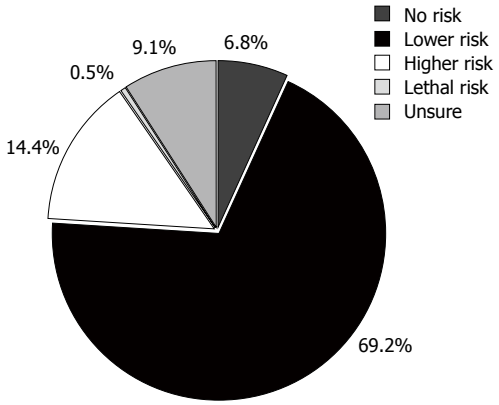

Figure 8 Physicians' perceptions of fecal microbiota transplantation risk.

examinations (92.7% and 90.9%) were all necessary considerations before qualifying as a donor.

DISCUSSION

The evolution of FMT has been rapid and certain. Physicians' and patients' awareness and perceptions of FMT are critical factors in determining FMT popularity. Our study was the first of its kind to investigate physicians' perceptions of FMT in an Asian country. Although there were only 844 physicians in our survey, which is a small proportion of the entire Chinese physician population (more than 200 millions), this survey covered most areas of China (22 out of 34 provinces) and was representative to some extent. This investigation will, undoubtedly provide information of FMT development in China and hopefully in other Asian countries.

Our investigation found high levels of FMT perception, as the vast majority of physicians had heard of FMT prior to this survey and nearly half of understood it well. Among these physicians, gastroenterologists had a better awareness and a more favorable attitude toward the development of this novel method than non-gastroenterologists, which was expected. All the physicians had a very high level of acceptance of FMT and a high interest in FMT training. In our study, geographical region was an important factor affecting physician perceptions of FMT. The significant geographical differences may be related to the differences in the economy, the frequency of information communication, and the uneven distribution of medical resources. Northwest China is less developed than other areas, and it has fewer medical resources and a slower spread of new knowledge and technology.

Chinese physicians' responses regarding the acceptance of FMT were somewhat astonishing. The high acceptance rate may be related to knowledge of Chinese traditional medicine in which FMT had originated. In this study, for the first time, the attitude of physicians toward FMT as an acceptable treatment was directly assessed. The results revealed that although human beings have a natural aversion to fecal material, the overwhelming majority of physicians were willing to accept FMT as a treatment method. A multivariate analysis revealed that increased awareness of FMT among physicians will enhance the likelihood of its acceptance. Conversely, the technique was less likely to be accepted by physicians working in higher level hospitals. It is possible that the higher level hospitals were more rigorous and cautious in the administration and implementation of new technologies.

Physicians accepted FMT as a treatment modality mainly on account of its effectiveness and safety, and they considered it an optional therapy for refractory diseases. This result was consistent with clinical studies, which reported that FMT was effective and safe in some diseases that were refractory to standard therapy or had shown frequent recurrence. Currently, there are few data about physicians' attitudes about FMT. In one investigation, 65% (83/135) of physicians had neither offered nor referred a patient for FMT, with the most common reasons being lack of appropriate clinical indication (33%), patients' acceptance or otherwise (24%) and institutional or logistical barriers (23%)[18]. In our investigation, the primary concern of Chinese physicians was the patients' acceptance, followed by the absence of guidelines and system and ethical constraints, similar to physicians overseas. This result suggests that the standardization and extension of FMT are imperative.

In addition to recurrent CDI, physicians showed interest in the use of FMT for many other diseases. Several studies have confirmed the astounding efficacy of FMT in the treatment of RCDI. Studies on IBD, IBS, and chronic constipation treatment with FMT followed suit. Further, FMT has a potential therapeutic value in non-GI diseases associated with gut flora, such as obesity, metabolic syndrome and chronic fatigue syndrome, which is based on preliminary case reports or animal experiments[22]. The results of our survey on the selection of potential FMT indications were consistent with these studies, although additional rigorous studies are needed to determine the efficacy of FMT for these diseases.

Until now, there is no evidence that stool material from related donors was better than that from unrelated donors. One argument for the use of related donors is that they are presumed to have shared gut flora exposures; however, they are also more likely to test for infectious disease markers than unrelated volunteer donors[23]. A long-term multicenter follow-up study showed that CDI cure rates were not influenced by the donor-recipient relationship[24], which provided grounds for the commercialization of frozen fecal microbiota and the development of FMT. Nevertheless, donors with different genders, ages, diets or lifestyles may have varying effects on the efficacy of FMT, which should be confirmed by further studies.

FMT is often delivered *via* the lower GI route, including *via* colonoscopy and retention enema, and/or

via the upper GI route, such as by gastroduodenoscopy, a nasoenteric tube and oral pills. To date, the optimal approach is still unclear, and approximately 75% of cases with RCDI are administered *via* the lower GI tract, and 25% *via* the upper GI tract[25]. A systematic review reported that FMT administered by colonoscopy had a higher cure rate (91%) than other routes for RCDI[26]. However, a recent RCT demonstrated a remarkable cure rate using the nasoenteric tube compared to colonoscopy[27]. Our results revealed that the vast majority of physicians (76.6%) preferred the lower GI tract with the primary argument that it may be easily accepted by patients psychologically. Another reason for the selection of the lower GI tract was that it may theoretically have a lower risk with easier colonization *in situ*, compared with the upper routes through which the small intestinal bacterial may overgrow and whether the stool suspension can reach the entire colon is unknown.

In terms of risk, although the majority of physicians in our survey considered FMT safe, an overwhelming majority of physicians suggested rigorous screening of donors to lessen the risk, including collection of a detailed disease history, and stool and blood examinations. Transient abdominal discomfort such as bloating, diarrhea and abdominal cramps have been observed after FMT and often disappeared within two days after treatment[24,25]. However, limited long-term safety data exist. Reports of concurrent infections after FMT treatment exist. Elizabeth *et al*[28] described a patient with refractory ulcerative colitis who acquired *cytomegalovirus* infection after FMT, which revealed a potential risk of FMT, although it was not confirmed whether the virus was directly from the donor. Cases involving *norovirus*[29], *S.typhi*, and *Blastocystishominis* infections have been reported. In our research center, despite rigorous screening, a patient developed an infection with two opportunistic pathogens, *Proteusmirabilis* and *Candidaalbicans* following FMT[30]. We still have limited knowledge of the impact of FMT on the intestinal flora and subsequent secondary infections after it. Therefore, the clinical utility of FMT must follow a strict and standardized protocol. It is recommended that patients undergo FMT in a hospital instead of at home. A standard protocol to screen donors is imperative.

In summary, this study is the largest survey of physicians' perceptions of FMT and it is the first time that physicians' perception of the indications, donors, and other technology associated with FMT have been evaluated in an Asian country. The keen interest, high acceptance and good understanding of FMT provide the grounds and conditions for the development of this novel treatment in China. The need to establish a standard procedure and protocol cannot be overstated.

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COMMENTS

Background

While there has been growing interest in fecal microbiota transplantation (FMT), it is still in early phases worldwide. Physicians' and patients' perceptions and attitudes toward FMT play an important role in determining its acceptability. This article explores Chinese physicians' perceptions towards FMT to provide information and an assessment of FMT development in China.

Research frontiers

There are a few reports discussing patients' attitudes towards the acceptance of FMT. Nevertheless, few studies exist regarding physicians' perceptions of this technique; all of these studies were conducted in Western countries. The acceptance of FMT in Asian countries remains unknown.

Innovations and breakthroughs

This is the first study to acquire physicians' perceptions of FMT in an Asian country. This study was representative with a large respondent number (844 eligible questionnaires were collected) and a vast coverage area of China (22 out of 34 provinces); thus, it can provide preliminary information for the FMT development in China. Additionally, the authors reviewed the literature and traced the history of human fecal medicine back 3000 years to the "Collection of 52 Prescriptions", and they found that the first use of human fecal suspension by mouth occurred 2nd century.

Applications

The keen interest and high acceptance of FMT provide the grounds and conditions for the development of this novel treatment in China. Nevertheless, guidelines and strict protocols are necessary to implement this technique.

Terminology

FMT refers to the instillation of fecal suspension from a healthy person into the gastrointestinal tract of a patient to cure a certain disease by restoring the construction of the intestinal flora.

Peer-review

The strongest point of this manuscript is being the first of its kind in China and other Asian countries. The idea is original and interesting, exploring the knowledge and attitudes regarding fecal microbiota transplantation (a very hot topic in gastroenterology nowadays) among Chinese physicians. The results give some ideas regarding how FMT might impact on clinical practice in the foreseeable future and provide important findings.

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META-ANALYSIS

Hybrid, sequential and concomitant therapies for *Helicobacter pylori* eradication: A systematic review and meta-analysis

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Abstract

AIM: To compare hybrid therapy (HT) with traditional sequential therapy (ST) and concomitant therapy (CT) for *Helicobacter pylori* (*H. pylori*) eradication.

METHODS: We performed an electronic search of PubMed, Embase, and the CENTRAL database. Randomized controlled trials (RCTs) of HT were included in the meta-analysis. The primary outcome was the eradication rate of *H. pylori*. The secondary outcomes included the compliance rate and adverse event rate. Effect estimates were pooled using the random-effects model.

RESULTS: Twelve studies were included. Pooled results showed no significant differences in eradication rate between HT and ST in per-protocol (PP) analysis (RR = 1.03, 95%CI: 0.94-1.12, P = 0.59) or in intention-to-treat (ITT) analysis (RR = 1.00, 95%CI: 0.89-1.12, P = 0.94). HT and ST showed similarly high compliance rate (96% *vs* 98%, P = 0.55) and acceptable adverse event rate (30.3% *vs* 28.2%, P = 0.63). No significant results were seen in the eradication rate between HT and CT in PP analysis (RR = 1.01, 95%CI: 0.96-1.05, P = 0.76) or in ITT analysis (RR = 0.99, 95%CI: 0.95-1.03, P = 0.47). HT displayed a slightly higher compliance rate than CT (95.8% *vs* 93.2%, P < 0.05). The adverse event rates of HT and CT were similar (39.5% *vs* 44.2%, P = 0.24).

CONCLUSION: Compared with ST or CT, HT yields a similar eradication rate, high compliance rate, and acceptable safety profiles.

Key words: Hybrid therapy; Sequential therapy; Concomitant therapy; *Helicobacter pylori*; Meta-analysis

Core tip: This meta-analysis of randomized controlled trials compared the novel hybrid therapy with sequential and concomitant therapy in the treatment of *Helicobacter pylori*. The eradication rate, compliance rate and the adverse event rate were investigated as the main outcomes and were compared. Overall, similar results were shown regarding these outcomes by hybrid and sequential therapy, and by hybrid and concomitant therapy. Hybrid therapy could be an effective and safe alternative to sequential or concomitant therapy.

Song ZQ, Zhou LY. Hybrid, sequential and concomitant therapies for *Helicobacter pylori* eradication: A systematic review and meta-analysis. *World J Gastroenterol* 2016; 22(19): 4766-4775 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i19/4766.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i19.4766

INTRODUCTION

Approximately 50% of the global population are infected with *Helicobacter pylori* (*H. pylori*). The presence of *H. pylori* in the stomach is directly associated with a series of gastric diseases, including chronic gastritis, peptic ulcer, and gastric cancer[1]. Triple therapy, consisting of one proton pump inhibitor (PPI), amoxicillin, and clarithromycin, has been established as the standard first-line treatment for *H. pylori* eradication since the 1997 Maastricht Conference[2]. However, the eradication rates have decreased to unacceptable levels (less than 80%) in many countries[3]. Growing resistance of *H. pylori* strains to clarithromycin and metronidazole is the major cause of treatment failure[4,5].

Worldwide efforts led to the development of new regimens to improve the eradication rate. Sequential therapy is one of the latest innovations, which was introduced by Zullo *et al*[6] in 2003. It entails the use of a PPI and amoxicillin for the first 5-7 d, followed by 5-7 d of PPI-clarithromycin-metronidazole (or tinidazole)[2,3]. With less clarithromycin resistance[3], the sequential regimen was more effective than standard triple therapy for *H. pylori* eradication[7,8]. However, some researchers argued that the benefit of sequential therapy only resulted from additional antibiotic therapy. Thus, it has been postulated that the four components of sequential therapy could be administered concurrently as concomitant therapy comprising PPI-clarithromycin-amoxicillin-metronidazole over several days[9]. The latest guideline recommends sequential and concomitant therapies as alternative first-line treatment in areas with a high rate of clarithromycin resistance[2].

Hybrid therapy entails administration of amoxicillin and a PPI for 5-7 d, followed by a PPI, amoxicillin, metronidazole, and clarithromycin for 5-7 d[10]. The recent randomized clinical trials (RCTs) of hybrid therapy showed conflicting results. Two studies showed that hybrid therapy outperformed sequential therapy in *H. pylori* eradication[11]. However, similar eradication rates were presented by other studies[12-14]. Furthermore, the duration of sequential or concomitant therapy was inconsistent between the studies. Therefore, we conducted this meta-analysis to evaluate the efficacy of hybrid therapy. We compared the efficacy, compliance, and safety of this new therapy with sequential or concomitant therapy.

MATERIALS AND METHODS

Search strategy

This meta-analysis was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) Statement[15]. Two reviewers independently performed systematic literature search of PubMed, Embase, and the Cochrane Central Register of Controlled Trials (CENTRAL) from their inception through October 2015. The search strategy is shown in Table 1. We used the following keywords or MESH Terms: "*helicobacter pylori*" or "*H. pylori*", "hybrid" or "sequential-concomitant". The language was limited to English. We also manually searched the references of eligible studies in case of any omission.

Inclusion criteria

Studies meeting the following inclusion criteria were included in the meta-analysis: (1) comparison of hybrid therapy (proton-pump inhibitors and amoxicillin for 5 to 7 d, followed by proton-pump inhibitors, amoxicillin, clarithromycin, and metronidazole for another 5 to 7 d) with other treatment regimens (sequential therapy, concomitant therapy, or triple therapy) in patients with *H. pylori* infection, or comparing different durations of hybrid therapy; (2) randomized controlled trials (RCTs); (3) *H. pylori* infection was diagnosed with rapid urease test, 13C-urea breath test, histology, or culture; and (4) comparison of the eradication rate, compliance, and/or adverse events. The *H. pylori* eradication was assessed by UBT at least 4 wk after treatment.

Data extraction and quality assessment

Two authors independently abstracted the data using a standardized form. The following data were collected from each study: author and year, study design, country, sample size, gender, comparison arms, diagnosis of *H. pylori,* eradication of *H. pylori*, and follow-up. The quality of the included study was evaluated by the Jadad scale, which assessed the study quality by randomization (2 points), blinding (2

Table 1 Characteristics of included studies involving hybrid therapy

Ref.	Region	Design	No. of patients	Age, mean or range, yr	Men, %	Hybrid group	Control group	Confirmation of infection	Confirmation of eradication	Follow-up	Jadad score
Hsu *et al*[10] (2011)	Taiwan	Single-arm	117	54	50	E 40 mg + A 1g, bid, 7d; E 40 mg + A 1g + C 500 mg + M 500 mg, bid, 7d	NA	RUT, UBT, and histology	UBT	8w	NA
Sardarian *et al*[25] (2012)	Iran	RCT	420	43	48	P 40 mg + A 1g, bid, 7d; P 40 mg + A 1 g + C 500mg + T 500 mg, bid, 7d	Sequential therapy (P 40 mg + A 1g, bid, 5d; P 40 mg + C 500mg + T 500 mg, bid, 5d)	RUT and/or histology	UBT	8w	3
Molina-Infante *et al*[27] (2013)	Spain, Italy	RCT	343	18-87	49	O 40 mg + A 1g bid, 7d; O 20 mg +A 1 g + C 500 mg + N 500 mg, bid, 7d	Concomitant therapy (O 20 mg + A 1 g + C 500 mg + N 500 mg, bid, 14d)	UBT or any two of RUT, histology, or culture	UBT	8w	3
Zullo *et al*[6] (2013)	Italy	RCT	270	49	41	O 40 mg + A 1 g bid, 7d; O 20 mg + A 1 g + C 500 mg + T 500 mg, bid, 7d	Concomitant therapy (O 20 mg + A 1 g + C 500 mg + T 500 mg, bid, 5d); sequential therapy (O 20 mg + A 1 g, bid, 5d; O 20 mg + C 500mg + T 500 mg, bid, 5d)	RUT and histology	UBT	6w	3
Oh *et al*[13] (2014)	Korea	RCT	184	57	37	R 20mg + A 1g bid, 7d; R 20 mg + A 1 g + C 500 mg + M 500 mg, bid, 7d	Sequential therapy (R 20 mg + A 1g, bid, 7d; R 20 mg + M 500 mg, bid, Mo 500 mg, qd, 7d)	RUT or histology	UBT	6w	3
De Francesco *et al*[12] (2014)	Italy	RCT	440	47	42	O 20 mg + A 1g bid, 7d; O 20 mg + A 1 g + C 500 mg + T 500 mg, bid, 7d	Concomitant therapy (O 20 mg + A 1 g + C 500 mg + M 500 mg + T 500 mg, bid, 5d or 14d); sequential therapy (R 20 mg + A 1 g, bid, 7d; R 20 mg + M 500 mg, bid, Mo 500 mg, qd, 7d)	RUT+histology	UBT	6-8w	2
Wu *et al*[23] (2014)	Taiwan	RCT	220	53	49	E 20 mg + A 1g bid, 3d; E 20 mg + A 1 g + C 500 mg + M 500 mg, bid, 7d	Hybrid therapy (E 20 mg + A 1g bid, 5d/7d; E 20 mg + A 1 g + C 500 mg + M 500 mg, bid, 7d)	RUT, UBT, histology, or culture	UBT or triple negative (RUT + histology + culture)	8w	3
Cuadrado-Lavin *et al*[28] (2015)	Spain	RCT	300	44	38	O 20 mg + A 1 g bid, 5d; O 20 mg + A 1 g + C 500 mg + M 500 mg, bid,5d	Concomitant therapy (O 20 mg + A 1g + C 500 mg + M 500 mg, bid, 10d)	RUT, UBT, or histology	UBT	4w	3
Heo *et al*[29] (2015)	Korea	RCT	422	57	59	E 20 mg + A 1g bid, 5d; E 20 mg + A 1 g + C 500 mg + M 500 mg, bid, 5d	Concomitant therapy (E 20 mg + A 1 g + C 500 mg + M 500 mg, bid, 10d)	Any two of UBT, histology, or RUT	UBT	4w	3
Hwang *et al*[26] (2015)	Korea	RCT	284	59	46	R 20 mg + A 1g bid, 7d; R 20 mg + A 1g + C 500 mg + M 500 mg, bid, 7d	Sequential therapy (R 20 mg + A 1g, bid, 7d; R 20 mg + M 500 mg, bid, Mo 500 mg, qd, 7d)	UBT, histology, or RUT	UBT	4w	3
Chen *et al*[11] (2015)	Taiwan	RCT	175	53	37	R 20 mg + A 1g bid, 7d; R 20 mg + A 1 g + C 500 mg + M 500 mg, bid, 7d	Sequential therapy (R 20 mg + A 1g, bid, 5d; R 20 mg + C 500 mg + M 500 mg, bid, 5d)	RUT + histology, culture	RUT + histology or UBT	8w	2
Metanat *et al*[24] (2015)	Iran	RCT	270	46	44	P 40 mg + A 1g, bid, 5d; P 40 mg + A 1 g + C 500 mg + T 500 mg, bid, 5d	Sequential therapy (P 40 mg + A 1 g, bid, 7d; P 40 mg + A 1 g + C 500 mg + T 500 mg, bid, 7d)	RUT, histology	UBT	8w	2

A: Amoxicillin; C: Clarithromycin; E: Esomeprazole; M: Metronidazole; Mo: Moxifloxacin; N: Nitroimidazole; O: Omeprazole; P: Pantoprazole; R: Rabeprazole; RUT: Rapid urease test; T: Tinidazole; UBT: 13C-urea breath test.

points), and attrition information (1 point)[16].

Statistical analysis

The effect size was calculated as the relative risk (RR) and the 95% confidential interval (CI) for each dichotomous outcome. The meta-analysis was conducted using the STATA software (StataCorp LP, College Station, TX, United States). The eradication rate, compliance rate and side effects rate were pooled by the Comprehensive Meta-Analysis statistical package (CMA Version 2.2, Biostat, Englewood, NJ, United States). The random-effects model using the DerSimonian and Laird method was employed for pooling the data because of suspected heterogeneity[17]. The heterogeneity was evaluated by the Cochran's Q statistic (statistical significance defined as $P < 0.05$), and the I^2 statistic (significant heterogeneity defined as $I^2 > 50\%$)[18]. Intention-to-treat (ITT) analysis was preferred to a per-protocol (PP) approach. The non-

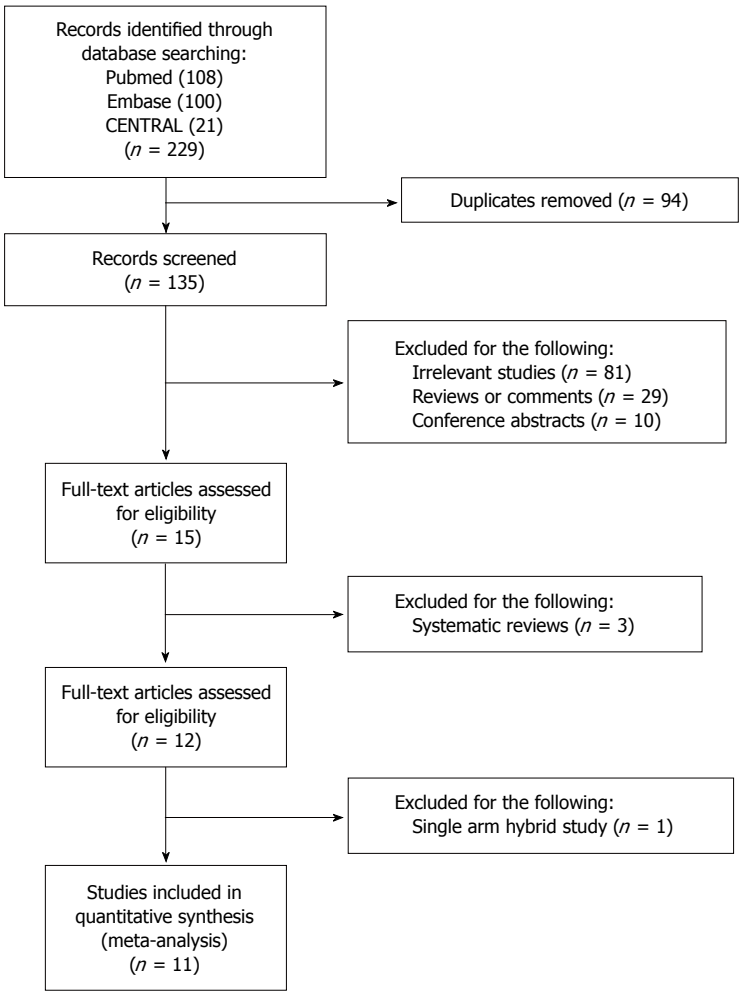

Figure 1 Study selection process of the meta-analysis.

compliant patients or withdrawals were included in the ITT analysis to minimize bias[19]. Sensitivity analysis was performed by excluding the studies one by one. Subgroup analyses were conducted by stratifying the duration of therapy. The publication bias was assessed by the Egger's test and the funnel plot. $P < 0.05$ was considered statistically significant.

RESULTS

Study selection

Our initial search identified 229 publications in total, including 108 articles from PubMed, 100 from Embase, and 21 from the CENTRAL database. Ninety-four duplicate publications were excluded. We discarded 81 irrelevant studies, 31 reviews or comments, and 10 conference abstracts. Fifteen records were eligible for full-text evaluation, of which one was a single-arm hybrid therapy study[20], and three were systematic reviews[4,21,22]. In the final meta-analysis, two studies compared different durations of hybrid therapy[23,24]. Six studies compared hybrid therapy with sequential therapy[11-14,25,26], and 5 studies compared hybrid therapy with concomitant therapy[12,14,27-29]. The selection process is shown in Figure 1. The characteristics of included studies are shown in Table 1. In the quality assessment, the blinding item was least fulfilled as no study used placebo or declared blinding to treatment regimen for patients or researchers. Except for three RCTs[11,12,24], most RCTs described the method of randomization. All studies clearly presented the follow-up data and conducted ITT analysis.

Overall eradication rate of hybrid therapy

The eradication rate was reported in 12 studies. In PP analysis, the overall eradication rate was 91.2% (88.5%-93.4%), with significant heterogeneity (I^2 = 63.9%, $P < 0.05$). In subgroup analyses, the pooled rate was 91.1% (87.4%-93.8%) for 10 studies using the 14-d regimen, and 90.3% (84.6%-94.1%) for 4 studies using the 10-d regimen (Figure 2A). In ITT analysis, the pooled eradication rate was 85.2% (82.1%-87.8%). For 10-d regimen (4 records) and 14-d regimen (10 records), the pooled rate was 82.2% (75.7%-87.2%) and 86.5% (82.6%-89.7%), respectively.

Different durations of hybrid therapy

Only two RCTs compared the hybrid therapies lasting 10 and 14 d, respectively[23,24]. In PP analysis, the 14-d

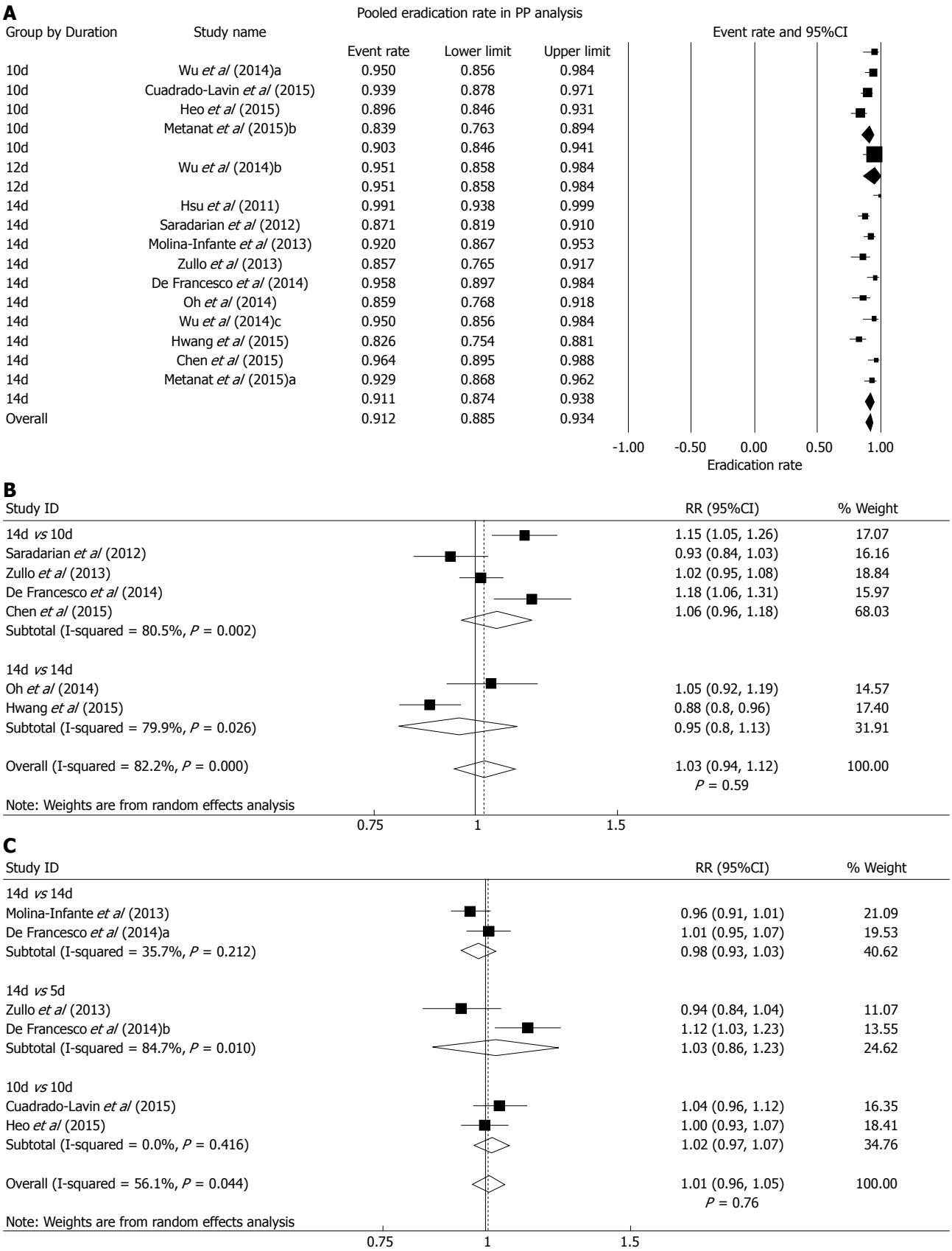

Figure 2 Per-protocol analysis. Forest plot showing the overall eradication rate of *Helicobacter pylori* (*H. pylori*) using hybrid therapy based on data from PP analysis. Subgroup analyses were conducted based on different durations of hybrid regimen. B: Forest plot comparing hybrid with sequential therapy in *H. pylori* eradication using data from PP analysis. Subgroup analyses were conducted based on different durations of sequential regimen. C: Forest plot comparing hybrid with concomitant therapy in *H. pylori* eradication using the data from PP analysis. Subgroup analyses were conducted based on different durations of concomitant regimen.

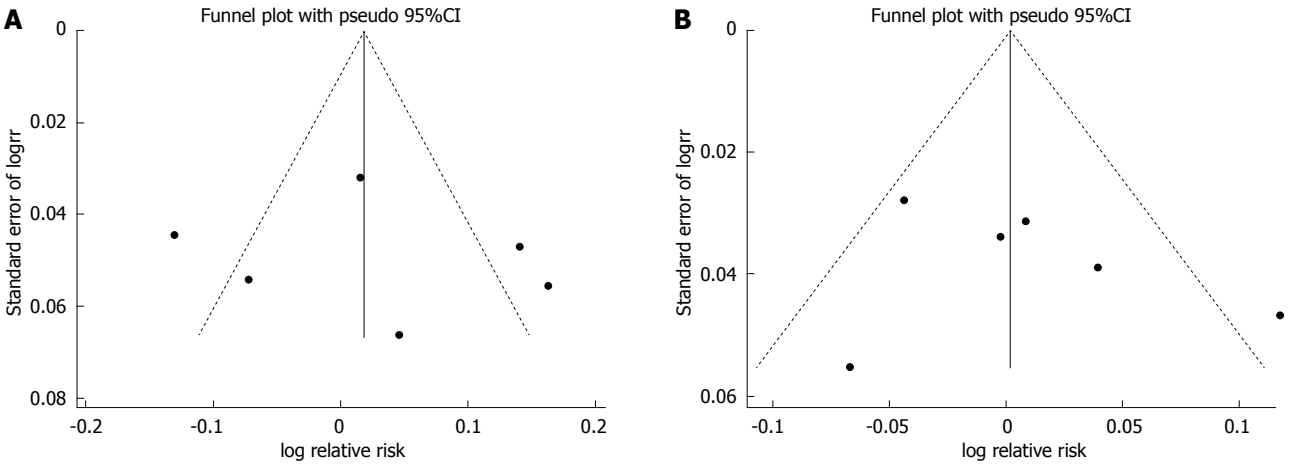

Figure 3 Publication bias. A: Funnel plot of studies comparing hybrid with sequential therapy; B: Funnel plot of studies comparing hybrid with concomitant therapy.

Table 2 Summary of meta-analyses: hybrid therapy *vs* sequential and concomitant therapy

Outcomes	Studies, *n*	Hybrid group	Control group	RR (95%CI)	I^2	*P* value for heterogeneity
Hybrid *vs* sequential						
Eradication rate (PP)	6	88.6%	87.8%	1.03 (0.94-1.12)	82.2%	< 0.05
Eradication rate (ITT)	6	84.3%	85.1%	1.00 (0.89-1.12)	85.2%	< 0.05
Compliance rate	5	96.0%	98.0%	0.99 (0.96-1.02)	50.4%	> 0.05
Side effect rate	6	30.3%	28.2%	1.05 (0.86-1.02)	37.8%	> 0.05
Hybrid *vs* concomitant						
Eradication rate (PP)	5	91.3%	92.4%	1.01 (0.96-1.05)	56.1%	< 0.05
Eradication rate (ITT)	5	84.8%	86.7%	0.99 (0.95-1.03)	0	> 0.05
Compliance rate	4	95.8%	93.2%	1.03 (1.00-1.05)[1]	0	> 0.05
Side effect rate	4	39.5%	44.2%	0.93 (0.82-1.05)	0	> 0.05

[1]Statistically significant results. ITT: Intention-to-treat; PP: Per-protocol.

regimen did not show significantly higher eradication rate compared with 10-d regimen (RR = 1.04, 95%CI: 0.92-1.18, $P > 0.05$). Significant heterogeneity was presented (I^2 = 73.4%, $P = 0.05$). In ITT analysis, no significant superiority was found for the 14-d regimen compared with the 10-d regimen (RR = 1.08, 95%CI: 0.99-1.19, $P > 0.05$), without heterogeneity (I^2 = 0%, $P > 0.05$).

Hybrid therapy vs sequential therapy

Eradication rate: Six studies were available[11-14,25,26]. Two Korean RCTs[13,26], and 2 Italian RCTs[12,14], were conducted by the same groups, during different study periods. In PP analysis, the eradication rate was 88.6% (95%CI: 83.6%-92.3%) for hybrid therapy and 87.8% (95%CI: 79.9%-92.9%) for sequential therapy. No statistically significant difference was found between the hybrid and sequential therapies, with significant heterogeneity (RR = 1.03, 95%CI: 0.94-1.12, $P = 0.59$; I^2 = 82.2%, $P < 0.05$) (Figure 2B). In ITT analysis, the eradication rate was 84.3% (95%CI: 79.3%-88.2%) for hybrid therapy and 85.1% (95%CI: 78.4%-89.9%) for sequential therapy. No significant differences were seen with hybrid therapy compared with sequential therapy (RR = 1.00, 95%CI: 0.89-1.12, $P = 0.94$). Significant heterogeneity was found (I^2 = 85.2%, $P < 0.05$) (Table 2).

Sensitivity analyses were carried out by excluding the studies one by one. Notably, no significant change was shown for PP or ITT results. Regarding sequential therapy, 4 studies used the 10-d regimen[11,12,14,25], and 2 studies used the 14-d regimen[13,26]. Based on the different durations, subgroup analysis of PP data did not find statistically significant changes for the 10-d regimen (RR = 1.06, 95%CI: 0.96-1.18) or for the 14-d regimen (RR = 0.95, 95%CI: 0.80-1.13) (Figure 2B). Similarly, subgroup analysis of ITT data revealed no significant alteration for the 10-d regimen (RR = 1.03, 95%CI: 0.88-1.20) or for the 14-d regimen (RR = 0.93, 95%CI: 0.79-1.09).

Compliance: Five studies evaluated the compliance[11,13,14,25,26]. Both therapies displayed a high compliance rate [96% (95%CI: 93%-98%)] for hybrid therapy, and 98% (95%CI: 95%-99%) for sequential therapy. No significant difference was observed (RR = 0.99, 95%CI: 0.96-1.02, $P = 0.55$; I^2 = 50.4%, $P > 0.05$) (Table 2).

Side effects: The overall adverse effect rate was 30.3% (95%CI: 20.9%-41.6%) for the hybrid therapy, and 28.2% (95%CI: 15.7%-45.4%) for the sequential

therapy. The hybrid therapy did not show significantly lower incidence of adverse effect (RR = 1.05, 95%CI: 0.86-1.02, P = 0.63). No significant heterogeneity was observed (I^2 = 37.8%, P > 0.05) (Table 2).

Hybrid therapy vs concomitant therapy

Eradication rate: Five studies were available[12,14,27-29]. In PP analysis, the eradication rate of hybrid and concomitant regimen was 91.3% (95%CI: 87.7%-93.9%) and 92.4% (95%CI: 89.2%-94.7%), respectively. In ITT analysis, the eradication rate of hybrid and concomitant regimen was 84.8% (95%CI: 78.9%-89.2%) and 86.7% (95%CI: 80.7%-91.0%), respectively. In PP analysis, no statistically significant difference was observed between hybrid therapy and concomitant therapy (RR = 1.01, 95%CI: 0.96-1.05, P = 0.76; I^2 = 56.1%, P < 0.05) (Figure 2C). In ITT analysis, no significant difference was found between the two regimens, and no heterogeneity was observed (RR = 0.99, 95%CI: 0.95-1.03, P = 0.47; I^2 = 0%, P > 0.05) (Table 2).

In sensitivity analysis by excluding studies one by one, no significant change was seen in PP or ITT analysis. For concomitant therapy, two studies presented results of the 14-d regimen[12,27], 2 of the 10-d regimen[28,29], and 2 of the 5-d regimen[12,14]. Subgroup analyses based on different durations of concomitant therapy revealed no significant difference.

Compliance: Four studies were relevant[14,27-29]. The compliance rate was 95.8% (95%CI: 93.2%-97.4%) for hybrid therapy, and 93.2% (95%CI: 89.7%-95.6%) for concomitant therapy. Patients receiving hybrid therapy showed significantly higher rate of compliance when compared with concomitant therapy (RR = 1.03, 95%CI: 1.00-1.05, P < 0.05). No heterogeneity was revealed (I^2 = 0%, P > 0.05) (Table 2).

Side effects: Four studies were included[12,14,27,28]. The overall side effect rate was 39.5% (95%CI: 21.7%-60.7%) for hybrid therapy, and was 44.2% (95%CI: 26.7%-63.2%) for concomitant therapy. No significant difference was seen between hybrid therapy and concomitant therapy (RR = 0.93, 95%CI: 0.82-1.05, P = 0.24). No heterogeneity was observed (I^2 = 0%, P > 0.05) (Table 2).

Publication bias

Publication bias was representatively evaluated for PP data. For hybrid *vs* sequential therapy, the funnel plot was symmetrical, with a non-significant result in Egger's test (P = 0.74) (Figure 3A). In hybrid *vs* concomitant therapy, the funnel plot was symmetrical (Figure 3B). No statistical significance was revealed by Egger's test (P = 0.48).

DISCUSSION

Eradication rate plays a pivotal role in evaluating the success of *H. pylori* treatment. The efficacy of *H. pylori* eradication was graded as follows: (1) excellent (> 95%); (2) good (90-95%); (3) fair (85-89%); (4) bad (81%-84%); and (5) unacceptable (< 80%)[30]. In ITT and PP analyses, therapeutic significance was achieved when the eradication rates exceeded 80% and 90%, respectively[26]. In this meta-analysis, hybrid therapy yielded a good eradication rate (91%) in PP analysis, and fair (85%) in ITT analysis, both exhibiting significant therapeutic values. The pooled data showed similar treatment success (an eradication rate closer to 90%) with hybrid, sequential, and concomitant therapies against *H. pylori.* Hybrid therapy had good compliance to medications, which was similar to sequential therapy and slightly better than concomitant therapy. The differences in adverse event rates were small between hybrid, sequential, and concomitant therapies. All the three therapies showed acceptable safety profile. The 10-d hybrid regimen did not show significant inferiority with respect to the eradication rate. Meta-analyses have shown that the eradication outcome was duration dependent[9]. However, the differences in eradication rate across all subgroups stratified by duration were minimal.

Currently, in the absence of any new drugs against *H. pylori,* different combination regimens, including sequential, concomitant, and hybrid therapies, have been investigated extensively. Hybrid therapy evolved from sequential therapy and concomitant therapy. Compared with sequential therapy, hybrid therapy extended the duration of amoxicillin. Prolonging the duration of traditional triple therapy from 7 to 10-14 d improved the eradication success rate by approximately 5%[2]. The prescription of PPI and amoxicillin was similar for concomitant and hybrid therapies. However, clarithromycin and metronidazole were used over a shorter duration of hybrid therapy. The adverse effects of metronidazole included nausea and regurgitation. Furthermore, both metronidazole and clarithromycin may cause bitter tastes[29]. With decreased pill burden, hybrid therapy was superior in cost-effectiveness over concomitant therapy.

The participants included in the RCTs were residents of Taiwan, Iran, Italy, Spain, and Korea, which represent regions with a high prevalence of antibiotic-resistant *H. pylori* strains[5,11]. Worldwide increase of *H. pylori* resistance to antibiotics, especially clarithromycin and metronidazole, is the most important determinant of eradication failure in traditional triple therapy[31]. For sequential therapy, the eradication rate of clarithromycin-resistant and metronidazole-resistant strains was 72.8% and 86.4%, respectively. However, the rate decreased to just 37% for dual-resistant strains[32]. Concomitant regimen outperformed sequential regimen in areas with a high incidence of clarithromycin and/or metronidazole resistance[33,34]. However, eradication was expected to fail (< 90%) when the prevalence of dual clarithromycin-metronidazole resistant strains was > 15%[34]. Compared with concomitant therapy, hybrid

therapy initially prescribed amoxicillin, which may prevent the occurrence of secondary clarithromycin resistance[35,36]. Compared with sequential therapy, hybrid regimen extended the duration of amoxicillin exposure. Hybrid therapy combined the advantages of sequential and concomitant therapy. Unfortunately, very few studies conducted antimicrobial susceptibility testing before hybrid treatment. Chen *et al*[11] showed that sequential therapy resulted in a 71.4% (5/7) eradication rate in patients harboring strains with dual resistance. Hybrid therapy yielded a 100% (4/4) eradication rate. Molina-Infante *et al*[34] revealed that for clarithromycin-resistant and dual-resistant strains, the concomitant regimen resulted in a 100% (8/8 and 3/3, respectively) eradication rate. By constrast, hybrid therapy only achieved a rate of 75% (6/8) and 33% (1/3), respectively. Nevertheless, the very small number of patients with resistant strains precluded definite conclusions.

Our meta-analysis represented the most comprehensive review of hybrid therapy and an update of two similar meta-analyses[21,22]. Notably, five studies have only recently been published, which were not included in previous meta-analyses[11,24,26,28,29]. The number of studies for meta-analysis doubled that of the previous studies, generating more robust conclusions, albeit with similar non-significant results between different regimens. Additionally, it was the first time that hybrid therapy was compared with different durations of sequential or concomitant therapy. The overall eradication rate with durations of hybrid therapy was demonstrated.

This study had several limitations. The number of included trials was still small, and the sample size was not large enough for the majority of studies. For example, although we did not detect the impact of different durations of sequential or concomitant therapy, the results should be extrapolated with caution as only few studies were included. Most RCTs did not report blinding to treatment regimen. Lack of blinding may influence compliance and the reporting of side effects. The quality of included studies was low. The majority of studies did not conduct susceptibility tests to determine antibiotic resistance[12,25,28]. In fact, we have tried to assess the eradication efficacy in resistant strains. However, we had insufficient related data and very small sample sizes of resistant patients. A number of confounding factors may play a role in determining the *H. pylori* eradication rates. Except for the disparity between different regions regarding the prevalence of resistant strains, the rates were influenced by genetic differences in the PPIs metabolism, degree of gastritis, administration of probiotics, and the nature of the underlying disease[37]. Additionally, different types of PPIs and nitroimidazole medications, and varying duration of follow-up may potentially generate small amounts of bias[27,28]. Participation in an RCT enhanced the patient compliance, and the compliance gap between hybrid therapy and other treatment regimens might be wider in clinical practice[29].

In conclusion, hybrid therapy yielded good eradication efficacy for *H. pylori* in regions with a high prevalence of antibiotic-resistant strains. Hybrid regimens achieved equivalent eradication rates compared with sequential or concomitant therapy. The compliance and adverse events were not different between hybrid, sequential or concomitant therapies. The 14-d and 10-d hybrid therapy showed similar eradication rates. Further studies are urgently required to clarify important differences in eradication of *H. pylori* in the setting of varying patterns of antibiotic resistance.

COMMENTS

Background

Previous trials reported inconsistent results regarding the efficacy, compliance rate and adverse events following the use of hybrid therapy when compared with traditional sequential therapy and concomitant therapy for the eradication of *H. pylori*.

Research frontiers

The emerging resistance of *H. pylori* strains is the major cause of treatment failure. Hybrid therapy represented a renewal of sequential therapy and concomitant therapy, and the efficacy and safety of hybrid therapy need to be investigated.

Innovations and breakthroughs

Our meta-analysis represented the most comprehensive review of hybrid therapy and an update of two similar meta-analyses. The authors for the first time, compared hybrid therapy following different durations of sequential or concomitant therapy. They also compared the different durations of hybrid therapy, and demonstrated the overall eradication rate of hybrid therapy.

Applications

Hybrid therapy showed a good eradication rate, high compliance rate, and acceptable safety profiles compared with traditional sequential therapy and concomitant therapy. These findings may represent a future strategy for the treatment of patients with *H. pylori* infection.

Peer-review

The study is a meta-analysis comparing hybrid therapy with traditional sequential therapy and concomitant therapy against *H. pylori* infection. The present manuscript included 5 additional studies published in 2015 and therefore strengthens the outcomes of previous meta-analyses.

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CASE REPORT

Endoscopic resection of sparganosis presenting as colon submucosal tumor: A case report

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Abstract

Human sparganosis is a rare parasitic disease caused by infection with the tapeworm *Sparganum*, the migrating plerocercoid (second stage) larva of *Spirometra* species. Sparganosis usually involves subcutaneous tissues and/or muscles of various parts of the body, but involvement of other sites such as the brain, eye, peritoneopleural cavity, urinary track, scrotum, and abdominal viscera has also been documented. Infections caused by *sparganum* have a worldwide distribution but are most common in Southeast Asia such as China, Japan, and South Korea. Rectal sparganosis is an uncommon disease but should be considered in the differential diagnosis of unusual and suspicious rectal submucosal tumors. We report a case of rectal sparganosis presenting as rectal submucosal tumor. We performed endoscopic submucosal dissection of the rectal submucosal tumor. The sparganosis was confirmed based on the presence of calcospherules in the submucosal layer on histological examination. Moreover, the result of the immunoglobulin G antibody test for sparganosis was positive but became negative after endoscopic submucosal dissection. Though rare, rectal sparganosis should be considered in the differential diagnosis of rectal submucosal tumor-like lesions. This case suggests that physicians should make effort to exclude sparganosis through careful diagnostic approaches, including detailed history taking and serological tests for parasites. In this report, we aimed to highlight the clinical presentation of *Sparganum* infection as a rectal submucosal tumor.

Key words: Rectum; Submucosal tumor; Sparganosis; *Sparganum*; Parasite disease

Core tip: This rare case exhibited the rectal sparganosis presenting as rectal submucosal tumor. This is the first case of rectal sparganosis presenting as submucosal tumor that are treated with endoscopic submucosal dissection. Though rare, this case suggests that sparganosis should be considered in the differential diagnosis of submucosal tumor-like lesions in gastrointestinal track.

Kim JK, Baek DH, Lee BE, Kim GH, Song GA, Park DY. Endoscopic resection of sparganosis presenting as colon submucosal tumor: A case report. *World J Gastroenterol* 2016; 22(19): 4776-4780 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i19/4776.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i19.4776

INTRODUCTION

Human sparganosis is a rare infectious disease caused by *Sparganum*, a plerocercoid tape worm larva of the genus *Spirometra*, as first described by Manson in 1882[1]. Infections caused by *sparganum* have a worldwide distribution but are most common in Southeast Asia such as China, Japan, and South Korea[2-4]. In humans, it is accidentally acquired by ingestion of larva-containing water or by eating raw snakes and frogs. Sparganosis usually involves subcutaneous tissues and/or muscles of various parts of the body, but involvement of other sites such as the brain, eye, peritoneopleural cavity, urinary track, scrotum, and abdominal viscera has also been documented[5,6]. The larvae are commonly found in the chest, abdomen, urogenital organs, extremities, central nervous system, and orbital region[5]. The rectum is a distinctly rare site of the infection. The rectum is a distinctly rare site of the infection. Clinical manifestations of sparganosis are diverse, including headache, non-specific discomfort, vague pain, palpable mass, or even no symptoms[7]. The most common clinical manifestation is a migrating subcutaneous mass. Clinically and radiologically, the mass may mimic a neoplasm. Thus, it is difficult to diagnose preoperatively in most cases[2]. We report an interesting rare case of rectal sparganosis presenting as a submucosal tumor (SMT) treated with endoscopic submucosal dissection (ESD). This report aimed to highlight the existence and clinical presentation of *Sparganum* infection, which is considered to be rare in South Korea. Moreover, this case report reveals an atypical picture where the larval form was not identified on gross examination of a resected specimen and the diagnosis was confirmed based on histological examination and enzyme-linked immunosorbent assay (ELISA) results.

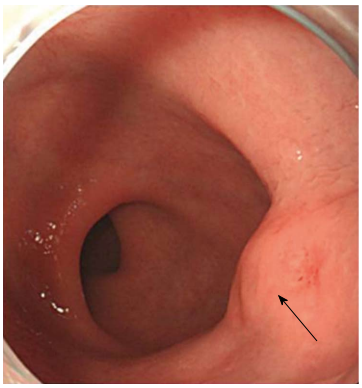

Figure 1 Endoscopic features of the rectal submucosal tumor. A prominently elevated submucosal tumor-like lesion covered with normal mucosa 5 cm above the anal verge (black arrow).

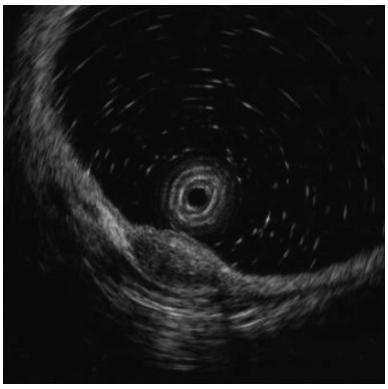

Figure 2 Endoscopic ultrasonographic features of the rectal submucosal tumor. An approximately 1.0 cm × 0.5 cm, hypoechoic lesion in the deep mucosa and submucosal layer.

CASE REPORT

In October 2013, a 38-year-old Korean male was referred to gastroenterology team for evaluation of rectal SMT. His medical history was uneventful. His family history was unremarkable. In addition, review of system and physical examination results were unremarkable. The results of the laboratory tests, including complete blood count, liver function test, renal function test, and coagulation factor assay, were normal. Moreover, no eosinophilia was observed, and tumor markers and anti-HIV antibody levels were normal. Colonoscopy revealed a round SMT covered with normal mucosa, located 5 cm from the anal verge (Figure 1). Endoscopic ultrasonography (EUS) revealed a well-demarcated hypoechoic mass 1.0 cm × 0.5 cm in size chiefly located in the deep mucosa and submucosal layer (Figure 2). For complete and total resection of the lesion and accurate histological diagnosis, we performed an ESD (Figure 3A and B). Histological examination of the resected specimen revealed chronic granulomatous inflammation with foreign bodies associated with acute suppurative inflammation (Figure 4A). A high-resolution image showed some calcospherules in the submucosal

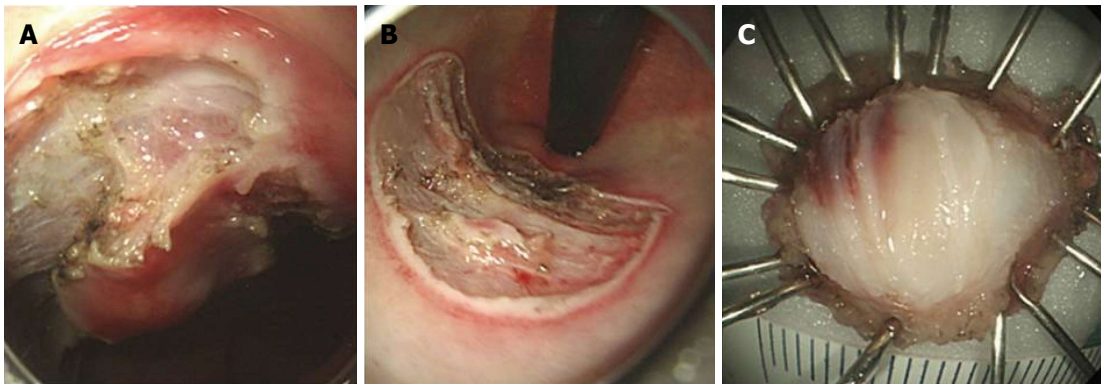

Figure 3 Rectal endoscopic submucosal dissection. A: During submucosal dissection; B: Endoscopic features after endoscopic submucosal dissection (ESD); C: Gross features of the specimen from ESD.

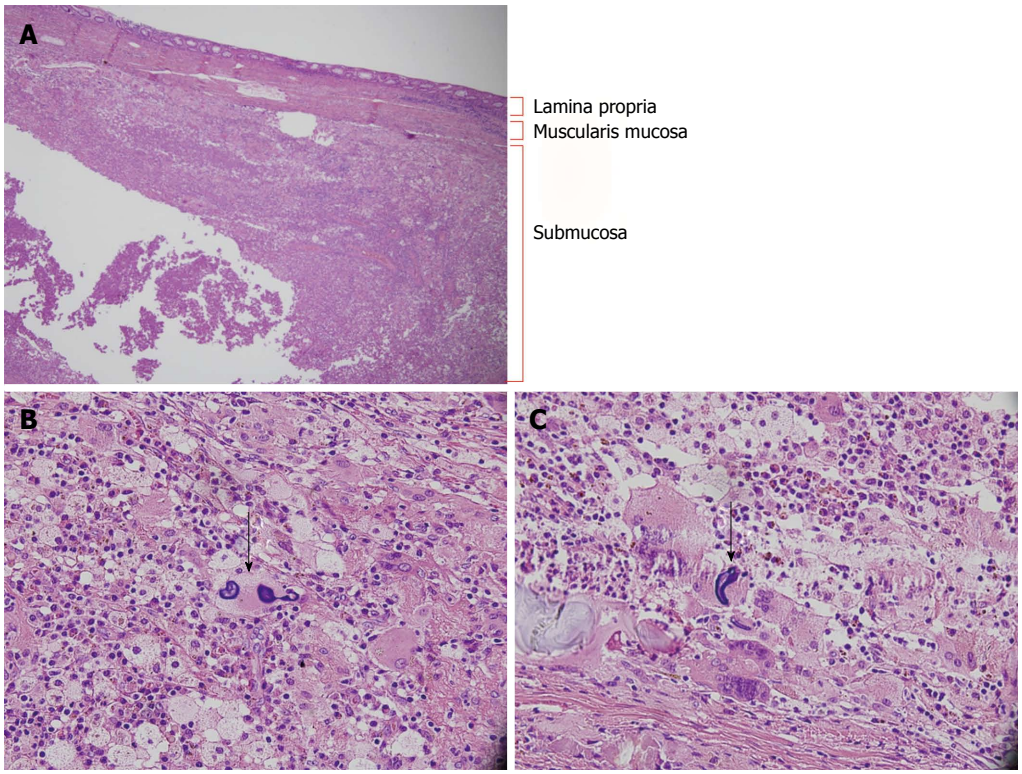

Figure 4 Pathological features. A: Suppurative inflammation and chronic granulomatous inflammation caused by foreign bodies mainly located in the submucosal layer (Hematoxylin-eosin stain, original magnification × 40); B and C: Calcospherules in the submucosal layer (Hematoxylin-eosin stain, original magnification × 100).

layer (Figure 4B and C). The calcospherules are characteristic structures that reveal previous parasitic infection. Subsequently, immunoglobulin G (IgG) antibody tests for cysticercosis, paragonimiasis, clonorchiasis, and sparganosis were performed, and the results were positive only for sparganosis. Although the gross examination of the excised tissue did not reveal any larval forms, the diagnosis was confirmed based on histological examination and ELISA results. We reevaluated the patient's medical history. Particularly noteworthy is the fact that he had enjoyed eating organic vegetables. We suggest that his dietary habit was the route of the infection. No additional therapy such as antiparasitic medication or surgery was administered. Thirteen months after the ESD, follow-up colonoscopy revealed only the presence of a scar at the site of the ESD, and chest radiography and abdominal computed tomography did not reveal any additional abnormal findings. Moreover, the result of the IgG antibody test for sparganosis became negative after the ESD. He was doing well at 24 mo of follow-up.

DISCUSSION

To our knowledge, this is the first case of sparganosis presenting as rectal SMT. Human sparganosis is a rare infectious disease caused by *Sparganum*, a

plerocercoid tapeworm larva of the genus *Spirometra.* Spargana larvae can be found in any part of the human body and have a preference for subcutaneous involvement and migration. In the extramammary organs, sparganosis most frequently manifests as a migrating subcutaneous nodule and presents clinically with vague or indeterminate symptoms.

Infections caused by *sparganum* have a worldwide distribution but are most common in Southeast Asia such as China, Japan, and South Korea. The most common route of infection is *via* ingestion of contaminated water containing procercoids, which can penetrate into the intestine and migrate to the muscle or subcutaneous tissue. The second route is from ingestion of raw or partially cooked frogs, snakes, fish, and chickens. The third route of infection may be from poultice applications infested with cyclops containing procercoids and are utilized for open wounds or the eyes[8]. Economic development and advancement in sanitation have influenced the routes, sites, and latent period of the infection. In our case, the patient denied eating raw meat or fish, including snakes and frogs. In addition, he had never traveled in Southeast Asia such as China, and Japan. Instead, he had enjoyed eating organic vegetables during his lifetime. We assume that the route of his infection was larvae from organic vegetables.

Sparganosis as a usual practice is diagnosed after the surgical removal of the worm from the site of inflammation. Although the larvae are decimated, the characteristic pathological findings of the chronic granulomatous inflammation with central necrosis and calcospherules can be helpful for diagnosis. However, in cases for which the feasibility of surgery is limited, surrogate diagnostic methods such as ELISA for *sparganum* in relation to a relevant history of exposure can be used[9]. ELISA performed for *sparganum*-specific IgG is highly sensitive and specific. Serum ELISA has 85.7% sensitivity and 95.7% specificity[6]. Negative ELISA results after surgical removal predict successful treatment of human sparganosis, but persistent positive reaction strongly suggests incomplete removal of the worm[8]. In our case where the gross examination of the excised tissue did not reveal any larval forms, the diagnosis was confirmed based on the histological examination and ELISA results. The negative ELISA result for sparganosis after ESD also strongly supported our diagnosis.

The definitive treatment of sparganosis is the surgical removal of larvae. However, when it cannot be removed surgically such as in multiorgan infection, drug therapy with praziquantel can be administered. However, many case reports showed that drug therapy with praziquantel did not have a favorable outcome and had high recurrence rates[10]. In our case, we did not administer praziquantel after ESD, the patient was doing well without recurrence for 24 mo after the ESD.

In conclusion, this is the first case of rectal sparganosis presenting as SMT. Furthermore, ESD performed to remove this parasite lesion is the first case. Though rare, rectal sparganosis should be considered in the differential diagnosis of rectal SMT-like lesions. This case suggests that physicians should make effort to exclude sparganosis through careful diagnostic approaches, including detailed history taking and serological tests for parasites. Although the ESD is a rather uncommon way in order to treat sparganosis, ESD can be considered for both diagnostic and therapeutic purposes like our case. In cases in which the rectal sparganosis is confined to the deep mucosa and/or submucosa on EUS and other sites are free of the disease, endoscopic resection with close follow-up should be considered as an alternative treatment modality.

COMMENTS

Case characteristics

38-year-old man with no significant medical history underwent colonoscopy for screening of the lower digestive tract. A rectal submucosal tumor was detected on 5 cm from the anal verge.

Clinical diagnosis

The patient had no symptom.

Differential diagnosis

Endoscopic ultrasonography (EUS) revealed a hypoechoic mass chiefly located in the deep mucosa and submucosal layer, which could be seen in the context of rectal neuroendocrine tumor, rectal tonsil, lymphoma, or chronic granulomatous inflammation.

Laboratory diagnosis

Laboratory data were within normal range with the exception of positive immunoglobulin G (IgG) antibody tests for sparganosis.

Imaging diagnosis

Colonoscopy revealed a round submucosal tumor covered with normal mucosa, located 5 cm from the anal verge. Endoscopic ultrasonography revealed a well-demarcated hypoechoic mass 1.0 cm × 0.5 cm in size chiefly located in the deep mucosa and submucosal layer.

Pathological diagnosis

The pathologic features of the resected specimen revealed chronic granulomatous inflammation with foreign bodies associated with acute suppurative inflammation and some calcospherules in high-resolution image.

Treatment

The patient underwent rectal endoscopic submucosal dissection for both diagnostic and therapeutic purpose.

Related reports

Human sparganosis is a rare infectious disease caused by *Sparganum*, a plerocercoid tapeworm larva of the genus *Spirometra.* Sparganosis usually involves subcutaneous tissues and/or muscles of various parts of the body, but rectal involvement presenting as submucosal tumor has not been documented.

Term explanation

A submucosal tumor is defined as any intramural growth under the mucosa, where etiology cannot readily be determined by endoscopy. EUS can be of help to make a diagnosis.

Experiences and lessons

Physicians should consider sparganosis in the differential diagnosis of rectal submucosal tumor-like lesions. And detailed history taking and serological tests for parasites can also be helpful in diagnosis.

Peer-review

This article is very interesting from the point of the parasitic disease because rectal sparganosis is an uncommon and rare. Furthermore, endoscopic submucosal dissection performed to remove this parasite lesion was probably the first case.

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CASE REPORT

Successful management of adult lymphoma-associated intussusception by laparoscopic reduction and appendectomy

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Author contributions: Hsu SD performed the surgery and provided the conception; Huang CY helped in interpreting the pathology; Chen YL helped in image interpretation; Lin YY and Tsuei YW helped edit the manuscript; and Yang TW collected data and wrote this manuscript.

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Informed consent statement: This case report is delivered for academic communication only, not for other purposes; In this case report, the images do not disclose the patient's personal information; Consent was acquired from the patient for publication of this case report.

Conflict-of-interest statement: The authors declare no conflicts of interest.

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Abstract

Although primary gastrointestinal lymphoma is a rare malignancy, it can cause an intussusception in adults and can be a clinically challenging condition to manage. Intussusception could progress to life-threatening complications if left untreated or could delay chemotherapy if inappropriate surgical management is used. We report a 31-year-old man diagnosed with human immunodeficiency virus who was being treated with antiretroviral therapy. He presented with nausea, vomiting, poor appetite, and intermittent, cramping abdominal pain for over 1 wk. Abdominal computed tomography revealed a well-defined homogeneous mass in the mesenteric root region, together with a long segmental wall thickening in the ileum with ileocolic-type intussusception, which was suspected to be caused by a lymphoma. The intussusception was successfully laparoscopically reduced, and the tumor involvement of the appendix was confirmed

by appendectomy with intraoperative frozen section. Systemic chemotherapy was immediately initiated after surgery without the need for bowel resection.

Key words: Intussusception; Adult; Intestinal lymphoma; Appendectomy

Core tip: In general, surgical management of adult with an intussusception mandates the resection of the involved bowel segment. However, the surgical resection of an intussusception that is caused by intestinal lymphoma is controversial because the intestinal involvement is generally diffuse. Concerning the diffuse invasive characteristics of gastrointestinal lymphomas, laparoscopic reduction of intussusceptions and appendectomy with intraoperative frozen section were both performed that enabled us to intraoperatively identify the tumor involvement of the resected appendix. By avoiding bowel resection, systemic chemotherapy could be initiated early after surgery.

Yang TW, Lin YY, Tsuei YW, Chen YL, Huang CY, Hsu SD. Successful management of adult lymphoma-associated intussusception by laparoscopic reduction and appendectomy. *World J Gastroenterol* 2016; 22(19): 4781-4785 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i19/4781.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i19.4781

INTRODUCTION

Although an intussusception is common in the pediatric population, it is a rare[1-5] but clinically challenging condition in adults, accounting for 1%-5% of mechanical bowel obstruction cases[6,7]. If left untreated, an intussusception could progress to tissue necrosis, bowel perforation, or peritonitis. In adults, it is typically because of the presence of a pathological lead point within the bowel, which is malignant in over half of all cases[6,7]. Most surgeons agree that adults with an intussusception require resection via surgery because most of such cases have lesions intraluminally. Nevertheless, the optimal resected range and if the intussusception should be reduced or not remain a controversy[8]. In this study, we present an unusual case of an adult with ileocolic intussusception that is caused by an intestinal lymphoma. He was successfully treated with laparoscopic surgery without bowel resection.

CASE REPORT

The patient was a 31-year-old man with human immunodeficiency virus (HIV) who was being treated with antiretroviral therapy. He presented with nausea, vomiting, poor appetite, and intermittent, cramping abdominal pain for over 1 wk. On arrival, his blood pressure was 126/66 mmHg; pulse rate, 92 beats per minute; and body temperature, 36.3 ℃. Physical examination disclosed tympanic sounds on percussion and mild periumbilical tenderness on palpation. Upper gastrointestinal (GI) panendoscopy was performed but revealed no specific findings; however, abdominal sonography subsequently identified a mass over the periumbilical region. Therefore, a contrast-enhanced computed tomography (CT) of the abdomen was performed, which revealed a well-defined, homogeneous mass, measuring approximately 6 cm × 4 cm × 7 cm, in the mesenteric root region (Figure 1, asterisk). The lymphoma was clinically highly suspected on the basis of imaging finding. In addition, there was evidence of a long segmental wall thickening in the terminal ileum with an ileocolic-type intussusception, and a lymphoma in the terminal ileum was also suspected (Figure 1, arrow).

The patient underwent diagnostic laparoscopy, which revealed tumors with diffuse involvement that included the terminal ileum and proximal colon. On

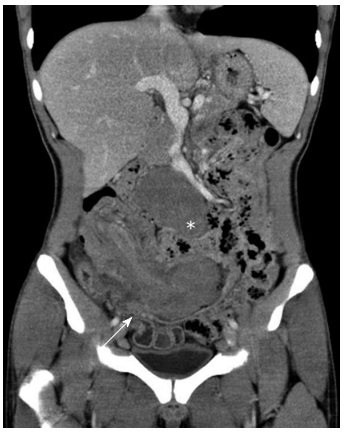

Figure 1 Contrast-enhanced computed tomography showed a well-defined homogeneous mass (asterisk) in the mesenteric root region, together with a long segmental wall thickening in the ileum with ileocolic-type intussusception (arrow).

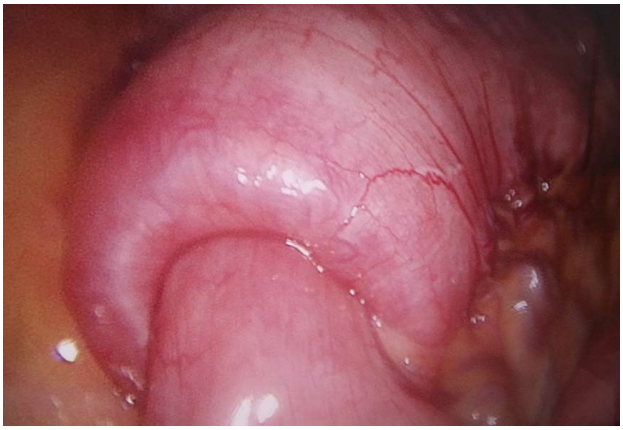

Figure 2 Invagination of the terminal ileum into the proximal colon.

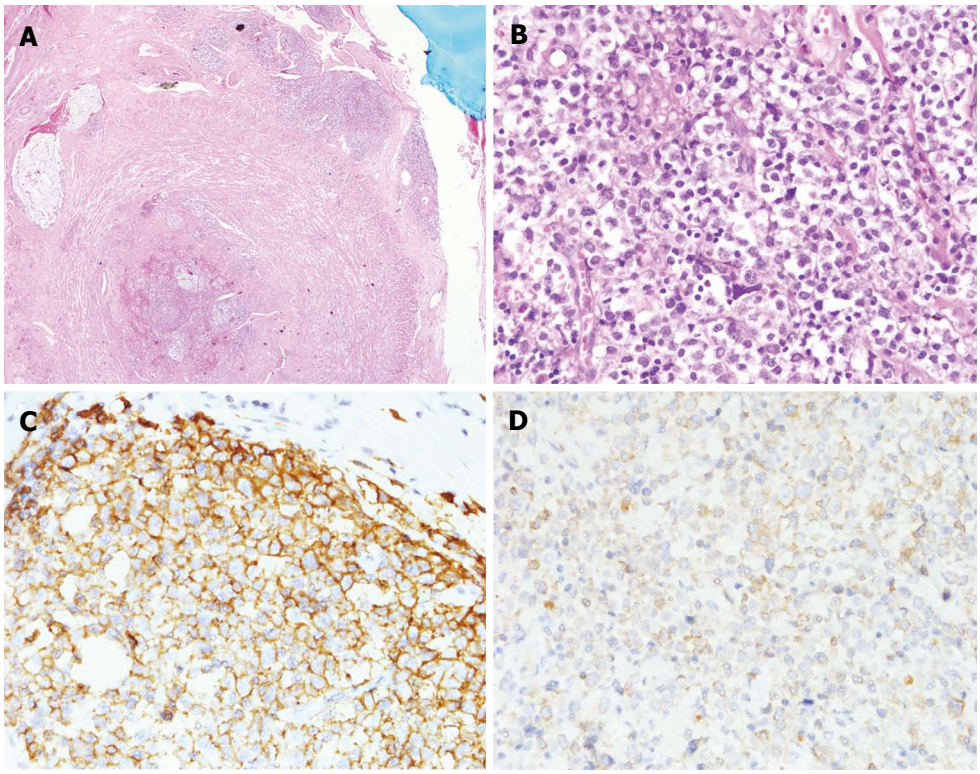

Figure 3 Histological and immunohistological examination of the specimens showing diffuse large B-cell non-Hodgkin's lymphoma. A: Magnification × 20; HE staining. Tumor cell infiltrates can be observed in the serosal layer of the appendix; B: Magnification × 400, HE staining, polymorphic large B cells; C: Magnification × 400, CD20(+); D: Magnification × 400, Bcl-2(+). Bcl-2: B-cell lymphoma 2; HE: Hematoxylin and eosin.

closer inspection, the terminal ileum and appendix were both invaginating into the proximal colon, and the tip of the appendix was still visible outside the invagination. Both of the invaginated ileum and proximal colon were relatively healthy, with no ischemic changes (Figure 2). We used non-traumatic graspers to retract the ileum and residual appendix and successfully reduced the intussusception. The appendix appeared red and swollen under laparoscopy.

Tumor cell infiltration in the serosal layer of the appendix was confirmed by an immediate frozen section of the appendix, and the final pathology result indicated a diffuse large B-cell lymphoma (Figure 3). The patient received systemic chemotherapy on postoperative day 12, and enteral feeding was well tolerated. The patient was subsequently discharged with good bowel movements and without any complication. He was referred to hematology for further chemotherapy treatment.

DISCUSSION

The optimal management of an intussusception remains controversial in adults. Surgical management usually involves the resection of the affected bowel to determine the underlying cause, which is malignant in more than half of all cases[9]. In contrast, chemotherapy is often the preferred treatment for primary intestinal lymphomas; surgery plays a limited role because the diffuse involvement would necessitate complete resection that might be complicated with short bowel syndrome[10-13]. Therefore, it is recommended that resection be reserved for patients with acute complications, such as obstruction, hemorrhage, abscess, or perforation, with no evidence supporting the uses of preventive surgery[14,15].

After Kaposi sarcoma, lymphomas are the second most malignancy that is observed in patients with HIV[16], and the most common type are diffuse large B-cell non-Hodgkin lymphoma[17]. GI tract involvement of lymphoma is common in patients with HIV[18], including the appendix, which is considered a part of the gut-associated lymphoid tissue. Leukemic and lymphomatous tumor involvement of the appendix can be primary or secondary[19]. Typical CT findings of the intestinal lymphoma include a enormous mass, extenseive infiltration fat planes preservation, multiple site involvement, and associated bulky lymphadenopathy[20,21]. Other differential diagnoses similar to these CT findings include carcinoids, adenocarcinomas, sarcoma (*e.g.*, gastrointestinal stromal tumor), and leiomyomatosis peritonealis disseminata. In our case, there was a well-defined homogeneous mass in the mesenteric root region and a long segmental wall thickening in the terminal ileum on contrast-enhanced CT of the abdomen. Intestinal lymphoma was presumed on the basis of this imaging, and tumor involvement of the appendix

was suspected because of its proximity to the terminal ileum. During surgery, the use of a frozen section of the resected appendix helped us quickly confirm that there was tumor-cell infiltration of the appendix. Given these findings, surgical management was limited to laparoscopic reduction of the ileocolic intussusception and appendectomy. Therefore, possible complications from diffuse large bowel resection could be avoided. Had the frozen section of the resected appendix not revealed tumor involvement, we might have considered performing a tumor biopsy instead.

Most intussusceptions are of the ileocolic type and occur because of a leading point that is located in the ileocolic region[1]. Because of this, we used traction of the appendix with surgical forceps to make the ileocolic intussusception easier to reduce and help perform the laparoscopic appendectomy.

Although adjuvant chemotherapy is typically initiated within 6-8 wk after surgery, several recent meta-analyses have confirmed that delayed administration of adjuvant chemotherapy is associated with significantly reduced overall survival[22,23]. Laparoscopic surgery is typical considered as a less-injurious surgery that enables earlier initiation of adjuvant chemotherapy[24,25]. Our patient was able to initiate systemic chemotherapy without too much delay because the surgery was limited to laparoscopic reduction of the intussusception and appendectomy.

In conclusion, diffuse large B-cell lymphomas may involve multiple sites in the GI tract and can cause intussusception in patients with HIV. To minimize any surgery-related complications and to improve prognosis, surgeons could consider laparoscopic reduction and appendectomy with intraoperative frozen section to identify tumor invasion. This approach facilitates earlier initiation of postoperative chemotherapy and could improve patient outcomes.

COMMENTS

Case characteristics

A 31-year-old human immunodeficiency virus (HIV)-infected man who was being treated with antiretroviral therapy, presented with nausea, vomiting, poor appetite, and intermittent, cramping abdominal pain for over 1 wk.

Clinical diagnosis

Nausea, vomiting, and intermittent abdominal cramping pain with mild periumbilical tenderness.

Differential diagnosis

Gastroesophageal reflux disease, peptic ulcer disease, irritable bowel syndrome, small or larbe bowel obstruction, cholelithiasis or pancreatitis.

Laboratory diagnosis

All laboratory tests were within normal limits.

Imaging diagnosis

Computed tomography (CT) of the abdomen revealed a 6 cm x 4 cm x 7 cm well-defined mass in the mesenteric root region, and a long segmental wall thickening in the terminal ileum with an ileocolic-type intussusception.

Pathological diagnosis

Diffuse large B-cell lymphoma.

Treatment

Laparoscopic reduction and appendectomy with postoperative systemic chemotherapy.

Related reports

Intussusception in patients with HIV is often associated with lymphoma, and other causes including Kaposi sarcoma and opportunistic infection had been reported.

Term explanation

After Kaposi sarcoma, non-Hodgkin lymphomas are the second most malignancy that is observed in patients with HIV, and the most common type are diffuse large B-cell lymphoma.

Experiences and lessons

For adult lymphoma-associated intussusception with diffuse intestinal involvement, laparoscopic reduction and appendectomy with intraoperative frozen section could be an alternative to make a definite diagnosis and avoid bowel resection.

Peer-review

CT findings in our case are not specific to lymphoma and could evoke other differential diagnosis.

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von Willebrand factor antigen as a therapeutic target of portal hypertension in cirrhosis

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Abstract

Increased thrombotic potential within the liver sinusoids due to local endothelial production of von Willebrand factor antigen macromolecules could represent an additional therapeutic target of portal hypertension in patients with cirrhosis. In this case, anti-inflammatory and antithrombotic drugs could modulate portal pressure by preventing the formation of intrahepatic platelet-induced microthrombi.

Key words: von Willebrand factor antigen; Endothelial dysfunction; Treatment; Portal hypertension

Core tip: The purpose of this letter to the Editor is to comment on the potential contribution of increased intrahepatic levels of von Willebrand factor as an additional mechanism that could be related to increased portal pressure in patients with cirrhosis and propose drugs which could decrease portal pressure on the basis of von Willebrand factor's production or effects.

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TO THE EDITOR

We read with great interest the article by Garbuzenko[1] on the pharmacotherapy of cirrhosis associated portal hypertension (PH) on the basis of its pathogenetic mechanisms. We fully agree that the major advances that have been made the recent years in our understanding of the pathophysiology of PH need to be translated into novel therapeutic strategies for the reversal of increased portal pressure. In his review, the author highlighted intrahepatic endothelial dysfunction

(ED) and endotoxinemia associated with bacterial translocation (BT) as important targets of future treatment of cirrhosis associated PH. Indeed, a large body of evidence suggests that sinusoidal ED is a key mediator of the pathogenesis of increased intrahepatic vascular resistance *via* a number of mechanisms which synergistically result in decreased hepatic nitric oxide (NO) production[2,3]. On the other hand, BT-related exposure to bacterial products and activation of cytokine cascade, which increase along with the severity of cirrhosis, are thought to play a dual causal role in PH by inducing downstream effects on intrahepatic NO synthesis[3,4] while, in contrast, stimulate NO production in the splanchnic arterial bed with a subsequent increase in portal venous inflow[5].

Apart from NO, the platelet adhesive protein von Willebrand factor antigen (vWF-Ag) has been proposed as a valuable indicator of ED in patients with cirrhosis[6,7]. vWF-Ag is produced and released as ultralarge multimers by activated endothelial cells in several vascular ED disorders[8,9], including inflammatory states[10]. Interestingly, vWF immunostaining is usually positive in large vessels but negative in the sinusoidal endothelial cells in the normal state[11]. On the occurrence of cirrhosis the sinusoidal endothelial cell becomes positive for vWF[12,13], presumably in association with the capillarization of hepatic sinusoids[14]. Based on accumulating data, it can be suggested that vWF-Ag may be a factor which initially links BT-related inflammation and intrahepatic ED, and subsequently predisposes to portal microthombosis with possible clinical implications in future therapeutic approaches to PH.

Circulating vWF-Ag levels have been found to be markedly elevated in patients with cirrhosis. Similarly to BT-related inflammation, plasma levels of vWF-Ag are significantly correlated with the severity of liver disease and PH[7,13,15]. A previous report by Ferro *et al*[7] demonstrated that endotoxemia is strongly correlated with plasma levels of vWF-Ag in the setting cirrhosis. It is also known that on the occurrence of superimposed systemic inflammation in patients with cirrhosis, plasma levels of vWF-Ag increase according to the degree of inflammatory response[16]. In this regard, endotoxin in a dose-dependent manner[7], and inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1 and IL-8, have been shown to stimulate the release of vWF-Ag from activated endothelial cells[17,18]. Further, the administration of nonabsorbable antibiotics in patients with cirrhosis caused a significant decrease of vWF-Ag plasma levels concomitantly with the decrease of endotoxemia[7]. vWF-Ag is cleaved by the protease ADAMTS13, which is mainly synthesized in the liver[19], into smaller forms which are less potent than the macromolecules in mediating platelet adhesion and aggregation[20]. The inflammatory cytokines TNF-α, IL-4, and IL-8 have been found to suppress ADAMTS13 synthesis in hepatic stellate cells and endothelial cells[18,21], which may contribute to the reduced levels of ADAMTS13 reported in cirrhosis[22].

It can therefore be suggested that increasing BT-mediated inflammatory responses as liver disease progresses predispose to accumulation of vWF-Ag multimers within the liver microcirculation thus enhancing platelet adhesion and aggregation to the sinusoidal endothelium despite the thrombocytopenic conditions of cirrhosis. This could lead to intrahepatic formation of platelet-induced microthrombi, progressive occlusion of portal microvasculature, and intensification of PH. BT-related release of inflammatory cytokines, such as TNF-α and IL-1, could potentiate the prothombotic state produced by vWF-Ag macromolecules within the cirrhotic liver by downregulating hepatic synthesis of protein C[23]. Intrahepatic microthrombi have been demonstrated in patients with cirrhosis and have been associated with accelerated liver fibrogenesis[24], which could further increase portal pressure. Microvascular occlusion of portal vein branches by platelet-rich thrombi due to inflammation stimulated elevation of vWF-Ag levels and decrease in ADAMTS13 activity has also been implicated in the pathogenesis of non-cirrhotic intrahepatic PH[25].

From a clinical point of view, higher concentrations of vWF-Ag levels in plasma[7,13,15] and in liver tissue[13] have been related to more severe PH and increased incidence of decompensation in patients with cirrhosis. Further, we have recently demonstrated in these patients that high levels of thrombin-antithrombin complexes, as a marker of hypercoagulability, was independently associated with major PH-related events, such as new-onset ascites and variceal bleeding, which could be related to the presence of thrombogenic mechanisms operative within the cirrhotic liver[26].

Consequently, available data suggest that increased thrombotic potential within the liver sinusoids due to high concentrations of vWF-Ag macromolecules could represent an additional therapeutic target of PH in patients with cirrhosis. In this case, anti-inflammatory and antithrombotic drugs could modulate portal pressure by preventing the formation of intrahepatic platelet-induced microthrombi.

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EDITORIAL

When a liver transplant recipient goes back to alcohol abuse: Should we be more selective?

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Abstract

Alcoholic liver disease (ALD) is one of the most common indications for liver transplantation (LT). However, it has always remained as a complicated topic from both medical and ethical grounds, as it is seen for many a "self-inflicted disease". Over the years, the survival rate of transplanted patients has significantly improved. The allocation system and the inclusion criteria for LT has also undergone some modifications. Early LT for acute alcoholic hepatitis has been subject to recent clinical studies with encouraging results in highly selected patients. We have learned from studies the importance of a multidisciplinary evaluation of candidates for LT. Complete abstinence should be attempted to overcome addiction issues and to allow spontaneous liver recovery. Risk factors for relapse include the presence of anxiety or depressive disorder, short duration of sobriety pre-LT and lack of social support. The identification of risk factors and the strengthen of social support system may decrease relapse among these patients. Family counseling of candidates is highly encouraged to prevent relapse to alcohol. Relapse has been associated with different histopathological changes, graft damage, graft loss and even decrease in survival among some studies. Therefore, each patient should be carefully selected and priority is to continue to lean on patients with high probability of success. The ethical issue remains as to the patient returning to drinking after the LT, hindering the way for other patients who could have received the same organ.

Key words: Liver transplantation; Alcoholic liver disease; Alcoholic cirrhosis; Selection criteria; Relapses

Core tip: Alcoholic liver disease is one of the most common indications for liver transplantation (LT). The selection criteria of the majority of transplant programs require 6-mo of complete abstinence, with the aim to allow spontaneous liver recovery and to overcome addiction issues. The evaluation of LT candidates should be multidisciplinary with a strong emphasis in family and social support and a strong patient commitment of abstinence to prevent relapses.

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Liver transplantation (LT) has become an accepted therapy for some patients with end-stage liver disease. The use of LT for alcoholic liver disease (ALD) continues to be controversial from both medical and ethical point of view[1,2]. However, it remains a common indication for LT worldwide[1,3,4].

One of the strongest ethical arguments against LT for ALD is the probability of relapse. For a patient to be listed as candidate for LT, 6 mo of abstinence must be achieved in most liver transplantation centers. Studies differ in the validation of this "6-mo rule" as well as in the real impact that relapse to drinking could have on the transplanted liver[5-8]. Recent studies have shown similar survival rates among LT for ALD and other chronic causes of end-stage liver disease recipients[1]. Early transplantation for acute alcoholic hepatitis (AAH), for example, has promising results[9,10]. However, a special multidisciplinary approach for alcoholic patients pre- and post-LT should be pursue with a goal of complete abstinence when possible.

Ever since Starzl *et al*[11], reported in 1963 the first three successful cases of liver transplantations in humans, an interest in increasing the use of life-saving intervention has evolved. By 1968, these investigators, reported the results of seven patients, one of them with 1-year post-transplant survival[12]. The next decade was characterized by important advances in tissue preservation, surgical techniques, control of infections and advances in immunosuppressive therapy with decrease in tissue rejection[13]. By 1979 there were about 318 human LT reported worldwide. The majority of them, performed at the University of Colorado (United States) and at the University Hospital at Cambridge and King's College Hospital (United Kingdom)[14]. In 1979, 15 years after the first LT, the 1-year survival rate had improved from to 28% to 50%[13]. Years later, the Organ Procurement and Transplantation Network was established by the United States government in 1987, operating under the United Network for Organ Sharing (UNOS)[15].

After the experimental years and over the last decades, there have been several changes in liver transplant indications and allocation system (UNOS). Initially, priority allocation was established based on "sickest first", meaning ICU's patients with acute complications - acute esophageal varices, hepatorenal syndrome or portosystemic encephalopathy[15]. The original allocation system was based on the Child-Turcotte-Pugh score. This was later proven to be sub-optimal in predicting the mortality and prioritization of patients[15]. In 2002, the national UNOS adopted the model for end stage liver disease (MELD) allocation system[16]. The MELD was developed to screen for short-term prognosis, and prioritize candidates according to disease severity, based on serum creatinine, serum bilirubin, international normalized ratio of prothrombin time (INR) and serum sodium[17].

Given the geographical disparity in organ allocation as seen by the disparities in waiting list and differences between units of organ, in 2013, the "Share 35" policy was implemented. Such policy instructs to give priority to candidate recipients for LT with MELD > 35[18]. Following this implementation, the waiting list for patients with MELD > 35 decreased from 18 d to 9 d in the last 2 years[19].

Currently, the accepted indications for LT are: acute liver failure, cirrhosis (with complications), liver metabolic diseases with systemic manifestations and systemic complications of chronic liver disease[20]. The latest guidelines for LT emphasize the importance of a multidisciplinary evaluation process; hepatology evaluation, surgical evaluation, laboratory testing, cardiac evaluation, hepatic imaging, psychiatry, psychology or mental health professional consultation, social work evaluation, financial and insurance counseling and nutritional evaluation[20].

As noted, ALD accounts for the second most common indication for LT[3,21]. ALD comprises subclinical biochemical damage, fatty liver, steatohepatitis, fibrosis and cirrhosis that can end up in end stage liver disease[21,22]. Other alcohol-induced entities include AAH and hepatocellular carcinoma[3,21,22]. On alcohol-induced injuries, the current guidelines continue to enforce the minimum of 6-mo of abstinence, this time is required to allow addiction issues to be addressed and helps in allowing spontaneous liver recovery. For patients with cirrhosis, LT is recommended once complications (ascites, hepatic encephalopathy, variceal hemorrhage or hepatocellular dysfunction) results in a MELD score > 15[20]. An entity that requires special consideration is AAH, a syndrome presenting with abdominal pain, fever, jaundice and acute hepatic decompensation[3]. Without transplantation, the probability of death in this group of patients is high and 70%-80% die within 6 mo[9,23,24].

Significant controversy on LT for alcoholic hepatitis exist[9]. Mathurin and coworkers examined patients that were not responding to medical treatment and that underwent an early liver transplant. Those pati-

ents that received an early LT had a significant higher survival than the patients in the medical therapy group[9]. Despite the favorable results, it should be noted that all the patients in this study were carefully selected and that 90% of non-responders to medical treatment were excluded due to a predisposition to addiction or unfavorable social or familial profiles. One of the key inclusion criteria for the enrollment in this pilot study, was the patient agreement to adhere to total alcohol abstinence. After LT, 3 out of 26 had alcohol consumption (11.5%). The authors concluded that the low rate of alcohol relapse was probably related to the carefully selection of recipients. More recently, Im and associates conducted a similar study in the United States, where early LT, in highly selected patients with severe alcoholic hepatitis, resulted in improved outcomes[10].

The main concerns remain the high chance of alcohol intake relapse after LT, which has been reported from 7%-95%[25]. The significant differences among data can be explained by differences in the use of terms "recidivism" and "relapse", which some studies utilize to define any alcohol intake, and in others to define heavy drinking[1,3,26-28]. Relapse to "harmful drinking" has been reported in 8%-21% of LT recipients[7,8,29-31]. Occasional drinks "slips", may not cause a significant graft damage, but with a history of alcoholism, it would be difficult to predict if these so called "slips", could end up in complete relapse and harmful alcohol abuse[1,32,33].

In an attempt to predict this risk, several analyses have been done[29,34-36]. Yates and coworkers used the high-risk alcoholism relapse (HRAR) scale, which consisted of evaluating the duration of heavy drinking, usual number of daily drinks, and inpatient treatment due to alcohol consumption[37]. In another study, 387 LT recipients were retrospectively analyzed by De Gottardi *et al*[29], finding an 11.9% relapse (harmful alcohol consumption). The presence of anxiety or depressive disorder, duration of sobriety of less than 6 mo, elevated HRAR score and age, were among the factors associated with increased risk of alcohol relapse[29].

Alcohol-induced injuries to allografts have been well documented[8,28,38]. In a retrospective study, Rice and coworkers evaluated the association between relapse and graft damage[28]. In this study, any alcoholic relapse was associated with increased risk of damage to the transplanted liver and particularly heavy drinking was associated with allograft loss (P = 0.008)[28]. Although most studies have found evidence of liver damage among relapse patients, they differ in reference to alcohol relapse and mortality rates[27,38].

Despite the established criteria regarding the 6-mo rule of abstinence, the sobriety time before LT is a strong predictor of relapse among recipients[6]. While on the waiting list, mandatory blood alcohol levels, urinary ethyl glucuronide and assistance to alcohol addiction units (AAU) could be used as strategies to prevent relapses[26,39,40]. In addition, the support of an AAU within the LT center has showed to decrease the prevalence of alcohol relapse. Carbonneau *et al*[40] studied the incidence of drinking while on the LT waiting list. They randomly checked blood alcohol levels, and 17% of them were found to relapse on drinking alcohol while on the LT waiting list. The time of relapse ranged from 2-23 mo. Interestingly, the increase of random blood alcohol level measurements was related to a decrease in alcohol use. Patients may have had lower alcohol ingestion by the fear of being caught and withdrawn from the list[40].

Addolorato *et al*[26] implemented the presence of an AUU in the LT center. Patients who were follow-up at the AUU had a lower relapse than the patients who were not seen by this unit (16.4% *vs* 35.1% respectively).

LT as a therapeutic option for alcoholic liver disease continues to be controversial. Different ethical and medical opinions preclude it to be fully accepted. Organ allocation for patients in whom the liver damage is considered to be self-inflicted may not be well accepted[2,29,41]. Yet, this practice continues. This may be causing conflict with the public opinion and may result in an unfavorable change in willingness to donate[2,5].

In an effort to assess the opinion on allocation priorities for LT, Neuberger *et al*[42] conducted a survey based study among general public, family doctors and gastroenterologists. Among groups a hypothetical alcoholic man and a prisoner were found to have lower priority for liver transplant allocation[42].

It is clear that given the current organ shortage, priority should be given to patients with high probability of success. For ALD, complete abstinence should be sought to allow possible liver repair and avoid unnecessary LT. Abstinence pre and post LT may be reinforced by the implementation of strict clinical and laboratory screening for alcohol relapses and strong support groups. AAU and strong social support system along with closer follow-up post transplant may help in preventing relapse on alcohol. The selection criteria should play a strong emphasis of the family environment and social structure and family counseling and alcohol abstinence should be also sought from family members prior to transplanting the patient with alcoholic liver disease to prevent future relapse. In cases of AAH, more multi-center studies with larger samples are needed to make solid conclusions.

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TOPIC HIGHLIGHT

2016 Inflammatory Bowel Disease: Global view

Inflammatory bowel disease and cancer: The role of inflammation, immunosuppression, and cancer treatment

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Abstract

In patients with inflammatory bowel disease (IBD), chronic inflammation is a major risk factor for the development of gastrointestinal malignancies. The pathogenesis of colitis-associated cancer is distinct from sporadic colorectal carcinoma and the critical molecular mechanisms underlying this process have yet to be elucidated. Patients with IBD have also been shown to be at increased risk of developing extra-intestinal malignancies. Medical therapies that diminish the mucosal inflammatory response represent the foundation of treatment in IBD, and recent evidence supports their introduction earlier in the disease course. However, therapies that alter the immune system, often used for long durations, may also promote carcinogenesis. As the population of patients with IBD grows older, with longer duration of chronic inflammation and longer exposure to immunosuppression, there is an increasing risk of cancer development. Many of these patients will require cancer treatment, including chemotherapy, radiation, hormonal therapy, and surgery. Many patients will require further treatment for their IBD. This review seeks to explore the characteristics and risks of cancer in patients with IBD, and to evaluate the limited data on patients with IBD and cancer, including management of IBD after a diagnosis of cancer, the effects of cancer treatment on IBD, and the effect of IBD and medications for IBD on cancer outcomes.

Key words: Inflammatory bowel disease; Cancer; Anti-tumor necrosis factor; Immunosuppression; Chemotherapy; Radiation

Core tip: Patients with inflammatory bowel disease (IBD) and cancer represent a challenging population. Gastroenterologists and oncologists caring for patients with IBD and cancer are increasingly confronted with questions regarding the management of IBD after a diagnosis of cancer, and conversely, the management of cancer in patients with IBD. This review seeks to explore the characteristics, risks, and pathogenesis of cancer in patients with IBD, and to evaluate the data on patients with IBD and cancer, including the interaction between IBD and cancer treatment.

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INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are chronic inflammatory conditions of the gastrointestinal tract. Although the disease pathogenesis is not fully understood, inflammatory bowel disease (IBD) is characterized by chronic inflammation of the gastrointestinal tract in genetically susceptible individuals exposed to environmental risk factors. Together, IBD is estimated to affect more than 0.4% of Europeans and North Americans, a number that is expected to increase over time[1]. It is well recognized that patients with IBD are at an increased risk of developing colorectal cancer (CRC), primarily the result of chronic intestinal inflammation[2-4]. More recently, patients with IBD have also been shown to be at increased risk of developing extra-intestinal malignancies, thought to be a consequence of immunosuppressive therapies and an underlying inflammatory state[5].

As the population of patients with IBD grows and ages, there is an inevitable increase in the risk of cancer development. Moreover, many of these patients may require cancer treatment, including chemotherapy, radiation, and immunotherapy, and many may require further treatment for their IBD. The focus of this review is to evaluate the characteristics, pathogenesis, and risks of cancer in patients with IBD, and to explore the relationship between IBD and cancer treatment.

IBD AND RISK OF CANCER

Cancer secondary to chronic intestinal inflammation

In patients with IBD, chronic intestinal inflammation is the primary risk factor for the development of gastrointestinal malignancy. Cancers as a result of chronic intestinal inflammation include CRC, small bowel adenocarcinoma, intestinal lymphoma, anal cancer, and cholangiocarcinoma (Table 1)[6].

Table 1 Cancer secondary to chronic intestinal inflammation

Cancer type	Standardized incidence ratio
Colorectal cancer[3]	5.7 (95%CI: 4.6-7.0)
Small bowel adenocarcinoma[20]	27.1 (95%CI: 14.9-49.2)
Intestinal lymphoma[36]	17.51 (95%CI: 6.43-38.11)
Anal cancer[60]	Data not available
Cholangiocarcinoma[23]	916.63 (95%CI: 297.88-2140.99) in UC

UC: Ulcerative colitis.

The risk and pathogenesis of inflammation-associated cancer has chiefly been described in colitis-associated CRC. In a meta analysis, quantitative estimates of CRC risk in UC have been reported to be 2% after 10 years, 8% after 20 years, and 18% after 30 years of disease[3]. Moreover, studies of CRC in UC have noted a high concordance between CRC risk with the location and extent of disease, with a standardized incidence ratio (SIR) of 1.7 for proctitis, 2.8 for left-sided colitis, and 14.8 for pancolitis[7]. All of these studies support the strong association between inflammation and cancer development.

Patients with IBD develop colon cancer in a manner similar to well described sporadic molecular mechanisms including mutations in the adenomatous polyposis coli (*APC*) gene, aneuploidy, DNA methylation, microsatellite instability (MSI), activation of the oncogene *k-ras*, activation of *COX-2*, and mutation in tumor suppressor genes *DCC/DPC4*, and eventual loss of *p53* function[8]. However, underlying colonic inflammation changes the timing and sequence of these genomic changes, yielding a process of carcinogenesis that is faster and multifocal[4]. Contrary to sporadic cancers in which the dysplastic precursor is the adenomatous polyp, dysplasia in patients with IBD can be localized, diffuse, or multifocal[4,9].

Studies mapping genomic instability secondary to DNA aneuploidy in patients with IBD indicate that these cell populations became more widely distributed, occupying larger areas of colonic mucosa[9]. Over time, further subpopulations with increasingly unstable genomics arise and expand, representing a whole field change, marking the entire colon at risk for further carcinogenesis[9,10].

In terms of specific molecular mechanisms that differ between colitis-associated cancer and sporadic cancer, early mutation in p53 is thought to play a fundamental role. Changes in p53 have been found in up to 85% of colitis-associated cancers[11]. Furthermore, alterations in p53 have been observed in biopsies from inflamed mucosa in more than 50% of patients with UC who did not have cancer, indicating a significant role of inflammation in these mutations[12]. In addition, loss of APC, an early event in the development of

Table 2 Cancer secondary to immunosuppression

Increased risk under anti-metabolites	Increased risk under anti-TNFα	Increased risk under anti-metabolite with anti-TNFα
Non-Hodgkin lymphoma[33-35]	Melanoma[42]	Hepatosplenic T-cell lymphoma[38]
Acute myeloid leukemia and Myelodysplastic syndromes[61]		
Non-melanoma skin cancers (basal and squamous cell carcinomas)[39-41]		
Urinary tract cancers[62]		

TNF-α: Tumor necrosis factor alpha.

sporadic CRC, is less frequent and tends to occur later in colitis-associated cancer[13]. DNA methylation also differs with increased hypermethylation of several genes, including *hMLH1* and *p16*, occurring earlier and contributing to microsatellite instability[14].

The immune response and oxidative stress play a critical role in the initiation and progression of carcinogenesis, contributing to the aforementioned molecular mechanisms leading to cancer. The inflammatory microenvironment of IBD, consisting of a variety of immune cells, epithelial cells, stromal cells, cytokines, and chemokines, has many similarities to the microenvironment of cancers, suggesting similar inflammatory mediators and mechanisms that promote both IBD and cancer development[15]. These mediators, produced by inflammatory cells, include tumor necrosis factor alpha (TNF-α), ILs-1, 6, 12, 13, 17, 22, and 23[15]. The interaction between the signaling of these cytokines and immune response play a major role in inflammation and colitis-associated cancer.

The increased expression of several inflammation-associated genes in IBD, such as cyclooxygenase-2 (COX-2) and nitric oxide synthase-2 (NOS-2), have also been noted in colonic neoplasia[12]. It is thought that reactive oxygen and nitrogen species produced by inflammatory cells expressing these genes not only directly damage colonic epithelium, but also contribute to the genetic alterations driving carcinogenesis[9].

In addition, alterations in the microbiota contribute to colitis-associated cancer. In mouse models of colitis-associated cancer susceptible to inflammation or cancer, cancer did not develop when the mice were germ-free or treated with antibiotics[16,17]. Studies of the microbiota in patients with CRC have demonstrated varying populations of bacteria that differ from cancer-free controls, suggesting that the complex interaction between the host genome, colonic epithelial-cell receptors, and the luminal microbiota create an environment conducive to carcinogenesis. Stool samples derived from CRC patients had higher levels of *Fusobacterium*, *Enterococcus*, *Escherichia*, *Shigella*, *Klebsiella*, *Streptococcus*, and *Peptostreptococcus*, *Firmicutes*, *Bacteroidetes*, and a depletion of bacteria belonging to *Lachnospiraceae* family compared to controls[18,19]. Although we are just beginning to understand the association between specific gastrointestinal microbes and cancer, much remains unknown regarding the causes and effects of these relationships and how manipulating the microbiome may have therapeutic potential.

In addition to CRC, small-bowel adenocarcinoma, specifically ileal carcinoma, has been shown to be significantly associated with the severity and duration of CD, and it is 20 to 30 fold more common in patients with CD compared to the general population[20]. Moreover, it is often found in areas with previous or synchronous ileal dysplasia, suggesting that it may evolve in a similar manner to the molecular and immune mechanisms of CRC described above[21]. In addition, cholangiocarcinoma, when associated with UC-primary sclerosing cholangitis (PSC), yields a risk nearly 160 fold greater than controls, suggesting the inflammatory state of IBD-PSC may contribute to biliary carcinogenesis[22,23].

Cancer secondary to immunosuppression

Given that chronic inflammation underlies the disease state of IBD, medications that mitigate inflammation by suppression of the immune system represent the cornerstone of treatment. In addition to treating IBD, it is postulated that these medications, such as immunomodulators [thiopurines (azathioprine or mercaptopurine) or methotrexate] and biologic agents (TNF-α antagonists), may reduce the incidence of inflammation-associated cancer. However, given that immunomodulators and biologic agents act on the immune system, they may also promote carcinogenesis.

Thiopurines and methotrexate promote the development of cancer by a variety of mechanisms including direct alteration in DNA, activation of oncogenes, reduction in physiologic immunosurveillance of malignant cells, and impaired immune control of oncogenic viruses[24-26]. Less is known about the carcinogenic potential of biologic therapies that block TNF-α and existing molecular data is inconsistent. TNF-α has been shown to exhibit anti-tumor effects by initiating cellular apoptosis of malignant cells, but it is secreted by most tumors to facilitate cellular survival and enhance neoplastic proliferation as a pro-tumor inflammatory cytokine[27-29].

Several studies have indicated a risk of therapy-associated malignancies in IBD patients. Population-based cohort and meta-analyses have demonstrated that current use of thiopurines for IBD is associated with a 1.3 to 1.7 overall relative risk of cancer, which is reversible after withdrawal[30,31]. Current exposure to TNF-α antagonists has not been shown to be associated with an overall excess risk of cancer, but data is very limited[32]. Specific cancers thought secondary to long-standing immunosuppression in the setting of IBD include lymphomas, acute myeloid leukemia, myelodysplastic syndromes, skin cancers, and urinary tract cancers (Table 2).

For lymphoma, multiple studies have demonstrated incidence ratios of non-Hodgkin lymphoma following thiopurine exposure ranging from 1.6 to 37.5, with no excess risk attributed to IBD itself[33-35]. The exception to this is primary intestinal lymphoma, where duration and severity of CD play a primary role[36]. In the setting of thiopurines, most lymphoma is Epstein-Barr virus (EBV)-associated and thought to result from the loss of immune control of EBV-infected B lymphocytes[37]. Furthermore, there have been several cases of fatal early postmononucleosis lymphoma in young men who are previously seronegative for EBV[33]. In addition, Hepatosplenic T-cell Lymphoma, though very rare, is primarily associated with thiopurine exposure in combination with TNF-α antagonists in both adolescent and young males[38]. However, recent data suggests that there is no excess risk of lymphoma in patients with IBD exposed to TNF-α antagonists[32].

In a study from the Cancers Et Surrisque Associé aux Maladies inflammatoires intestinales En France (CESAME) cohort, the risk of myeloid disorders was not increased among patients with IBD or ongoing thiopurine treatment (SIR = 1.54, 95%CI: 0.05-8.54), but patients with past exposures to thiopurines had an increased risk of myeloid disorders (SIR = 6.98; 95%CI: 1.44-20.36)[31].

For skin cancers, there is substantial evidence that thiopurines increase the risk of basal cell and squamous cell carcinomas, collectively known as nonmelanoma skin cancers (NMSC)[39-41]. In another study from the CESAME group, an increased risk of NMSC was observed in the patients with IBD and associated with ongoing thiopurine exposure (HR = 5.9; 95%CI: 2.1-16.4) and past thiopurine exposure (HR = 3.9; 95%CI: 1.3-12.1)[41]. However, in a large retrospective cohort of patients with IBD, there was no excess risk of nonmelanoma skin cancer attributable to TNF-α antagonists[40]. In addition, studies have demonstrated an increased risk of melanoma in patients with IBD, with no increased risk associated with thiopurine exposure[40-42]. Conversely, patients exposed to TNF-α antagonists have been found to be 1.5 to 2 times more likely to develop melanoma to patients with IBD who were not exposed to TNF-α antagonists[32]. As such, thiopurines increase the risk of NMSC whereas TNF-α antagonists increase the risk of melanoma.

Secondary or recurrent cancer in patients with a history of cancer

Given the above-mentioned risks of immunomodulator and biologic-associated malignancy, patients with a history of cancer were excluded from clinical trials of TNF-α antagonists. Additionally, there is substantial data within the transplant literature indicating that immunosuppression, such as thiopurines and calcineurin inhibitors, increases the risk of new and recurrent malignancies in patients with a history of cancer[43,44]. As such, oncologists and gastroenterologists generally suspend immunosuppression for IBD after a diagnosis of cancer, both while undergoing cancer treatment and during remission from cancer. This approach may worsen IBD and even complicate appropriate cancer management. Although there is little data on patients with IBD and a history of cancer, there is emerging data regarding the management of IBD after a diagnosis of cancer.

In 17047 patients in the CESAME prospective observational cohort, exposure to immunosuppression was independently associated with the development of cancer with an adjusted HR of 1.9 (95%CI: 1.2-3.0)[31]. However, it did not increase the risk of new or recurrent cancer in patients with a history of cancer[31]. Given the limited number of patients with IBD and a history of cancer with subsequent exposure to immunosuppression in the cohort, this conclusion only applied to thiopurine exposure and no conclusions were drawn on anti-TNF-α therapies[31].

A similar study from the New York Crohn's and Colitis Organization (NYCCO) representing a consortium of 8 academic medical centers found that nearly 30% of patients with IBD and a history of cancer developed new or recurrent cancer[45]. However, exposure to TNF-α antagonists, antimetabolites, or the combination was not associated with an increased risk of new or recurrent cancer within 5 years following a diagnosis of cancer (Log-rank P = 0.14)[45]. Furthermore, after adjusting for the risk of recurrence of prior cancer, there was still no difference in risk of new or recurrent cancer between exposure groups (anti-TNF-α HR = 0.32, 95%CI: 0.09-1.09; anti-TNF-α with an antimetabolite HR = 0.64, 95%CI: 0.26-1.59; antimetabolite HR = 1.08, 95%CI: 0.54-2.15)[45].

In addition, data from NYCCO showed that duration of anti-TNF-α after a diagnosis of cancer was not associated with the risk or type of new or recurrent cancer[45]. Studies within the rheumatoid arthritis literature corroborate these findings with data demonstrating no difference in the development of new or recurrent cancer in patients with a history of cancer who were subsequently exposed to anti-TNF-α agents compared with those receiving disease-modifying anti-rheumatic drugs alone[46,47]. However, given small sample sizes, these studies often grouped different types of cancers together. In the NYCCO study for example, all solid malignancies, such as breast, prostate, and lung, were grouped together. This statistical approach may not reflect the natural biologic activity of carcinogenesis and the direct effects of immunosuppression on cancer development, limiting the ability to draw conclusions on specific cancers.

CANCER TREATMENT AND IBD

While data on the risk of new or recurrent cancer under immunosuppression in patients with IBD and

a history of cancer is limited, though increasing, considerably less is known regarding the effects of cancer treatment on IBD, and the effect of IBD and medications for IBD on important cancer outcomes.

Effect of cancer treatment on IBD

In a study from the Massachusetts General Hospital, 84 patients with IBD and extra-intestinal cancer were assessed for the effect of cancer treatment on the course of IBD[48]. The authors found that 66.7% of patients with active IBD at their cancer diagnosis experienced remission from IBD thought secondary to cytotoxic chemotherapy. Conversely, 17.4% of patients in remission from IBD at their cancer diagnosis experienced a flare during or within 5 years after their cancer treatment[48]. In the remission group, the authors found the risk of flare to be greatest among patients who received hormonal therapies (combination cytotoxic chemotherapy with adjuvant hormone therapy HR = 12.25, 95%CI: 1.51-99.06; hormone monotherapy HR = 11.56, 95%CI: 1.39-96.43). This suggests that hormonal therapies for cancer, such as breast and prostate, may increase the risk of IBD reactivation or counter the protective effects of cytotoxic chemotherapy[48]. A majority of patients with active IBD at their cancer diagnosis appeared to benefit from cancer treatment in the form of IBD remission, which was much more likely if the cancer treatment included cytotoxic chemotherapeutics and less likely if patients were treated with hormonal monotherapy[48].

In this cohort, there was no appreciable modification in IBD medications after a diagnosis of cancer. TNF-α antagonists were continued in three patients and the proportion of patients maintained on immunomodulators decreased slightly from 22% to 14% after a cancer diagnosis[48]. These data, however, were not compared to a control group of patients without chemotherapy or without cancer to assess whether patients with IBD and extra-intestinal cancer experienced a course of IBD different from patients without chemotherapy or cancer.

However, other studies have demonstrated a major modification in IBD medications after a diagnosis of cancer. In a study from a French clinical prospective database, a diagnosis of extra-intestinal cancer had a marked impact on the management of IBD, but was not associated with significant modifications in activity of IBD[49]. A diagnosis of extra-intestinal cancer led to some changes in therapeutic strategy, with a lesser use of thiopurines (19% *vs* 25%, $P < 0.001$) and an increased use of intestinal surgery (4% *vs* 2.5%, $P = 0.05$)[49].

Effect of IBD on cancer

Little is known regarding specific cancer outcomes in patients with IBD. Oncologists have generally been reluctant to administer pelvic irradiation in the setting of IBD, as the tolerance of pelvic irradiation in these patients is largely unknown. There exists only one study in the literature from Green *et al*[50] which retrospectively examined 47 patients with IBD and rectal cancer treated over a 34-year period (1960-1994) from the Mount Sinai Hospital, New York. The authors found a five-year overall survival rate of 42% and disease-free survival of 43%, which were comparable to results published for non-IBD-associated rectal cancer at that time, however, patients with high-grade tumors had statistically lower rates[50]. Complications, such as gastrointestinal morbidity or small bowel obstruction, were comparable to those reported in several large randomized trials of adjuvant chemoradiation therapy in rectal cancer arising in the general population[50].

In terms of chemotherapy and associated cancer outcomes, a small study on 8 patients with IBD and gastrointestinal malignancy showed that the most common gastrointestinal adverse event was diarrhea, with 38% of patients experiencing greater than 7 stools per day over baseline and/or fecal incontinence, all of which occurred in patients with CD[51]. Several studies have examined the effect of IBD medications on cancer outcomes. Multiple studies have demonstrated a role of anti-TNF-α in improving cachexia and increasing chemotherapy tolerance in patients with non-small cell lung cancer, renal cell carcinoma, and pancreatic cancer[52-54]. Moreover, in patients treated with TNF-α antagonists, the occurrence of cancer during treatment was not associated with a worse prognosis, and may even have a protective effect by reducing aggressive metastatic breast cancers at a cellular level[55,56].

Immunotherapies for cancer and immune-related colitis

Immunotherapy for cancer has shown promise in cases refractory to conventional treatment. However, unguided immune stimulation in cancer patients presents its own challenges. There are several reports of anti-cytotoxic T-lymphocyte-associated protein-4 antibodies used for melanoma, such as ipilimumab, and programmed cell death-1 receptor inhibitors used for melanoma and non-small cell lung cancer, such as and nivolumab, producing an immune-related colitis that is remarkably similar to IBD[57,58]. These medications, particularly when used in combination, result in clinical symptoms, endoscopic manifestations, and pathologic cellular infiltrates that emulate IBD. Fortunately, the majority of these cases respond to conventional treatments for IBD such as systemic corticosteroids, budesonide, and infliximab[57,58].

In a recent study, 50% of patients with advanced melanoma and baseline autoimmune disease, such as rheumatoid arthritis, IBD, and psoriasis, experienced either autoimmune exacerbations or immune-related adverse reactions when treated with ipilimumab[59]. These reactions were generally manageable with standard treatment including corticosteroids and infliximab[59]. As the field of immunotherapy for cancer

evolves, we may see an increase in immune mediated colitis, which highlights the important role for T-cell checkpoint inhibitors in exacerbating IBD or causing an IBD-like colitis.

CONCLUSION

Patients with IBD are at an increased risk of cancer secondary to long-standing intestinal inflammation and secondary to immunosuppressive therapies. As the population of patients with IBD ages, there is an increasing risk of cancer development. Many of these patients will require cancer treatment and many will require further treatment for their IBD.

Much research is being devoted to exploring the role of chronic intestinal inflammation from IBD in carcinogenesis, and the role of immunosuppressive medications used to treat IBD in the promotion and prevention of cancer. Despite these efforts, much remains unknown regarding the interaction between IBD, medications for IBD, and cancer treatment, and the risk of cancer recurrence in patients with IBD and a history of cancer.

Understanding the effects of chemotherapy, hormonal therapies, radiation, and surgery for cancer on IBD may help identify patients at the highest risk for disease exacerbation during and after specific cancer treatments, especially in those who may require re-initiation of immunosuppressive therapies for IBD. In addition, while retrospective data has demonstrated some evidence for the safety of immunosuppression in patients with IBD and a history of cancer, prospective data are needed to validate these findings. Furthermore, data is lacking regarding specific cancers, treatments, and risk of recurrence under varying immunosuppressive medications for IBD. More data will permit the development of evidence-based, quantitative risk-benefit models including cancer and IBD-related variables to assist clinicians in managing this complex patient population.

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TOPIC HIGHLIGHT

2016 Inflammatory Bowel Disease: Global view

Hydradenitis suppurativa and inflammatory bowel disease: An unusual, but existing association

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Abstract

Inflammatory bowel disease (IBD) could be associated with several extra-intestinal manifestations (EIMs) involving musculoskeletal, hepatopancreatobiliary, ocular, renal, and pulmonary systems, as well as the skin. In the last years, hidradenitis suppurativa (HS) is acquiring an increasing interest. IBD, especially Crohn's disease (CD), is among the most reported associated diseases in HS patients. The aim of this paper is to give a brief overview of data showing a possible epidemiologic and pathogenetic association between IBD and HS. We performed a pooled-data analysis of four studies and pooled prevalence of HS in IBD patients was 12.8%, with a 95%CI of 11.7%-13.9%. HS was present in 17.3% of subjects with CD (95%CI: 15.5%-19.1%) and in 8.5% of UC patients (95%CI: 7.0%-9.9%). Some items, especially altered immune imbalance, are generally involved in IBD pathogenesis as well as invoked by HS. Smoking is one of the most relevant risk factors for both disorders, representing a predictor of their severity, despite, actually, there being a lack of studies analyzing a possible shared pathway. A role for inheritance in HS and CD pathogenesis has been supposed. Despite a genetic susceptibility having been demonstrated for both diseases, further studies are needed to investigate a genetic mutual route. Although the pathogenesis of IBD and HS is generally linked to alterations of the immune response, recent findings suggest a role for intestinal and skin microbiota, respectively. In detail, the frequent finding of *Staphylococcus aureus* and coagulase-negative staphylococci on HS cutaneous lesions suggests a

bacterial involvement in disease pathogenesis. Moreover, microflora varies in the different cutaneous regions of the body and, consequently, two different profiles of HS patients have been identified on these bases. On the other hand, it is well-known that intestinal microbiota may be considered as "the explosive mixture" at the origin of IBD despite the exact relationship having not been completely clarified yet. A better comprehension of the role that some bacterial species play in the IBD pathogenesis may be essential to develop appropriate management strategies in the near future. A final point is represented by some similarities in the therapeutic management of HS and IBD, since they may be controlled by immunomodulatory drugs. In conclusion, an unregulated inflammation may cause the lesions typical of both HS and IBD, particularly when they coexist. However, this is still a largely unexplored field.

Key words: Hydradenitis suppurativa; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Intestinal microbiota; Skin microbiota; Immunosuppressant drugs

Core tip: The present topic outlines the main data regarding a possible association between hydradenitis suppurativa and inflammatory bowel disease with particular attention to epidemiology, etiopathogenetic factors, genetic susceptibility, intestinal/skin microbiota and therapeutic analogies. Finally, an unregulated inflammation leading to microscopic granulomatous wounds may cause the lesions typical of both diseases, particularly when they coexist. However, this is still a largely unexplored field, and further studies are required.

Principi M, Cassano N, Contaldo A, Iannone A, Losurdo G, Barone M, Mastrolonardo M, Vena GA, Ierardi E, Di Leo A. Hydradenitis suppurativa and inflammatory bowel disease: An unusual, but existing association. *World J Gastroenterol* 2016; 22(20): 4802-4811 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i20/4802.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i20.4802

INTRODUCTION

Inflammatory bowel disease (IBD) is a group of chronic inflammatory conditions of the alimentary tract, that are mainly represented by Crohn's disease (CD) and ulcerative colitis (UC)[1]. These disorders could be associated with several extra-intestinal manifestations (EIMs) involving musculoskeletal, hepatopancreatobiliary, ocular, renal, and pulmonary systems, as well as the skin. In particular, joint, liver, eye, and skin EIMs are considered the most relevant and frequent manifestations[2].

Joint involvement is the most common EIM of IBD[2] and includes peripheral arthropathy, subclassified in pauciarticular, polyarticular forms, and axial arthropathy, such as sacroileitis and spondilitis. Primary sclerosing cholangitis represents the most common cause of hepatobiliary involvement in IBD patients, especially in UC[3,4]. Ocular complications, including episcleritis, scleritis and uveitis, occur more frequently in patients with isolated small intestinal CD[2].

Different dermatological manifestations may arise during the course of IBD. Indeed, pyoderma gangrenosum, psoriasis, Sweet's syndrome, aphthous stomatitis can be observed, even if erythema nodosum represents the most common IBD-associated dermatological disease. Moreover, in recent years, hydradenitis suppurativa (HS) has been acquiring an increasing interest, even though it may be frequently misdiagnosed as a consequence of an inadequate expertise[5].

HS[2] is defined as "a chronic inflammatory, recurrent, debilitating follicular skin disease that usually presents after puberty with painful deep seated inflamed lesions in the apocrine gland-bearing areas of the body, most commonly the axillae, inguinal and anogenital regions"[3]. HS diagnosis is based on the following clinical criteria: (1) the presence of typical lesions, (2) their characteristic sites, and (3) the chronic course of disease, showing recurring flares[5]. Hurley classification identifies three progressive stages of disease severity: (1) abscess formation, single or multiple, without sinus tract and scarring; (2) recurrent abscesses, with tract formation and healing wound, as well as single or multiple widely separated lesions; and (3) diffuse or multiple interconnected tracts and abscesses across entire area[4].

IBD, especially CD, is among the most reported comorbid diseases in HS patients[5].

Patients with HS and CD have more often been found to be smokers, and more likely to develop perianal disease, and to show an increased need for immunosuppressants and surgical resections[6]. Moreover, on the basis of recent evidence supporting the role of immune imbalance in both conditions[1-3,5-8], a shared pathogenesis between IBD and HS may be presumed. Indeed, multiple predisposing factors could influence the onset and progression of both diseases, *i.e.*, gut luminal agents, genetics and environmental factors[2].

The aim of this paper is to give a brief overview of data showing a possible epidemiologic and pathogenetic association between IBD and HS.

EPIDEMIOLOGY AND POOLED-DATA ANALYSIS OF LITERATURE

The first series of patients with both CD and HS was described by Church *et al*[9]. Twenty-four patients were recruited. The diagnosis of CD pre-dated that of

Table 1 Case reports about the association Crohn's disease-hydradenitis suppurativa

Ref.	*n*	Localization of CD	Localization of HS	CD predates HS
Ostlere *et al*[11], 1991	3	Colon	Anogenital	NR
Burrows *et al*[12], 1992	2	Colon	Anogenital, axillae, groin	NR
Gower-Rousseau *et al*[13], 1992	1	Ileo-colon	Perineum	NR
Attanoos *et al*[14], 1993	3	Colon, ileo-colon, colon-jejunum	Anogenital, axillae, perineum	Yes
Tsianos *et al*[15], 1995	1	Colon	Anogenital, axillae, sternum	Yes
Roy *et al*[16], 1997	1	Ileo-colon	Axillae	NR
Martínez *et al*[17], 2001	1	Ileo-colon	Axillae	Yes
Roussomoustakaki *et al*[18], 2003	1	Ileo-colon	Anogenital, axillae, groin	No
Yazdanyar *et al*[19], 2010	2	Colon	Axillae, groin, submammary	No
Goertz *et al*[20], 2008	1	Colon	Perianal	Yes
dos Santos *et al*[21], 2012	1	Rectum	Perianal	Yes
Hiraiwa *et al*[22], 2013	1	Ulcerative colitis	Groin	Yes

CD: Crohn's disease; HS: Hydradenitis suppurativa.

HS by an average of 3.5 years. More recently, other 15 patients with CD and HS followed at Mount Sinai Medical Center in the period 2003-2013 have been reported[10]. Apart from these few cohort studies, only case reports about association of IBD-HS have been published. Such single cases are summarized in Table 1[11-22].

Currently, the prevalence of HS in IBD has been investigated in four studies[23-26]. In the pilot one[23], 158 patients with IBD were asked by a standardized questionnaire about the presence of symptoms suggestive of HS, such as recurrent painful boils in the axillae and/or groin[27]. Further, a picture representing a classical HS skin lesion was shown to the patients in order to have a visual comparison with the injury they were suffering from. On the basis of this method, HS prevalence of 16% in patients with IBD was detected (17% and 14% in CD and in UC patients, respectively). The same authors replicated this study in a larger sample (1093 IBD patients), with an overall prevalence of 23%, in detail 26.3% for CD and 18.3% for UC[24]. A female predominance and a correlation between smoking and severe HS course were recorded. More recently, two other epidemiological studies were carried out. In a cohort study performed in the Olmsted county in Minnesota[25], 679 IBD patients were followed up over a median period of 19.8 years. In such patients, the clinical diagnosis of HS was directly established by dermatologists. HS was found in 8 patients (1.8%), 5 with CD and 3 with UC. A significant association with obesity, female sex and perianal CD disease was found. Two out of 3 subjects with UC had undergone ileal pouch-anal anastomosis. Compared with the control group, the incidence rate ratio of HS in IBD was 8.9 [95% confidence interval (CI): 3.6-17.5]. The 10- and 30-year cumulative incidence of HS was 0.85% and 1.55%, respectively. Axillae, groin, and thighs were the most common sites of involvement. Finally, Janse *et al*[26] showed an HS prevalence of 10.6% (134 out of 1260) in their IBD cohort, with a higher association with CD (15.1%) than with UC (6.1%). In this study, the diagnosis was achieved using a questionnaire validated for HS[27].

We performed a pooled-data analysis of the four cited studies, as shown in Figure 1. The pooled prevalence of HS in IBD patients was 12.8%, with a 95%CI of 11.7%-13.9%. HS was present in 17.3% of subjects with CD (95%CI: 15.5%-19.1%) and in 8.5% of UC patients (95%CI: 7.0%-9.9%), thus confirming a stronger association with CD. In three out of four studies, the diagnosis of HS was established by means of a questionnaire, and these three studies showed the highest prevalence rates. This detail may lead to the conclusion that such diagnostic strategy, despite validated, could overestimate the prevalence of HS in comparison to the clinician direct evaluation.

The clinical pattern of the IBD-HS association appears to be characterized by female predominance, increased frequency of tobacco smoking and by the fact that intestinal disease foregoes skin involvement. Clinical and pathogenetic features of HS and IBD association are summarized in Table 2.

PATHOGENETIC FACTORS

The pathogenesis of HS is still obscure. Ever-growing attention has been focused on the role of the immune system, and recent findings suggest the involvement of the interleukin (IL)-23/Th17 pathway in HS-related inflammatory response[28].

HS is characterized by epidermal alterations such as psoriasiform epidermal hyperplasia and keratin pluggings. In HS lesions, the epidermis is an active source of proinflammatory cytokines. It shows inflammasome activation and can be stimulated by IL-17$^+$ cells. The inflammatory process in HS involves the recruitment of innate immune cells, particularly IL-17-expressing neutrophils[29].

Impaired Notch signalling has been proposed to be a crucial pathomechanism of HS, capable of compromising apocrine gland homoeostasis and leading to subsequent stimulation of TLR-mediated innate immunity[30]. This mechanism has been hypothesized not only as an inducer of inflammation in

Table 2 Main clinical and pathogenesis features of hydradenitis suppurativa and inflammatory bowel disease (adapted from van der Zee *et al*[23])

	CD	UC	HS
Localization	Entire alimentary tract	Colon	Inverse areas of the skin
Layer of inflammation	Transmural	Mucosa	Deep derm
Confluency of lesions	No (skip lesions)	Yes	Yes
Fistulae	Yes	No	Yes
Influence of smoking	Aggravates	No (or improvement)	Aggravates
Disease chronicity	Yes	Yes	Yes
Genetic predisposition	Yes	Yes	Yes
Influence of microbiota	Yes	Yes	Yes
Female predominance	↑	↑	↑↑
Response to anti-TNFα therapy	Yes	Yes	Yes

CD: Crohn's disease; UC: Ulcerative colitis; HS: Hydradenitis suppurativa; TNFα: Tumor necrosis factor alpha.

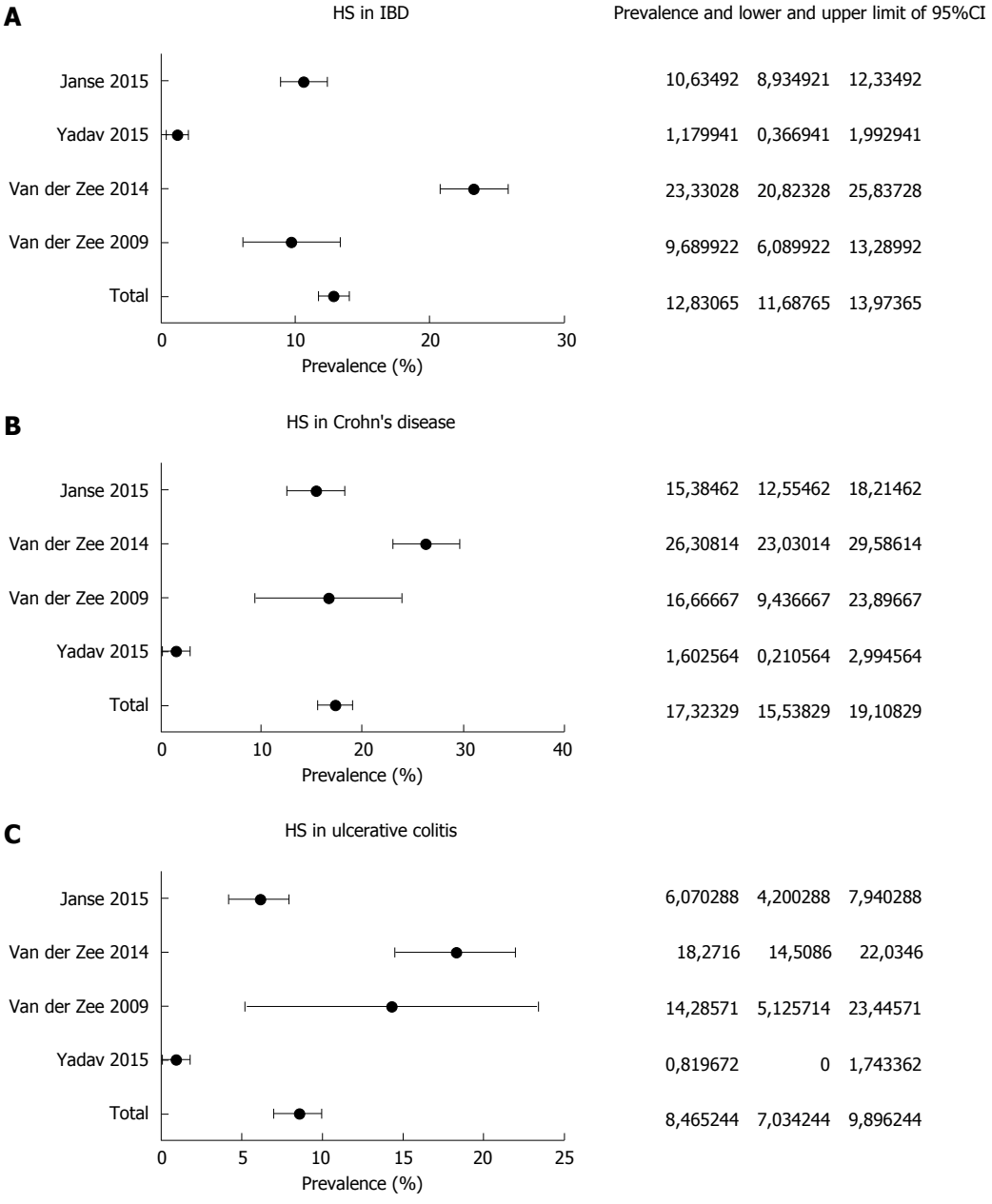

Figure 1 Pooled-data analysis of studies exploring the prevalence of Hydradenitis suppurativa in subjects with inflammatory bowel disease (A), either Crohn's disease (B) and ulcerative colitis (C). CD: Crohn's disease; IBD: Inflammatory bowel disease; HS: Hydradenitis suppurativa.

HS but also as responsible for an insufficient feedback regulation of overstimulated innate immunity, linking HS to other Th17-driven comorbidities.

On the other hand, an alteration of immune imbalance with a prevalence of inflammatory cytokines has been clearly stated for inflammatory bowel disease and, at the moment, strongly affects therapeutic approach[1-4].

Some items, generally involved in IBD pathogenesis, are invoked also for HS.

Smoking

Smoking is one of the most relevant risk factors for both HS and CD, representing a predictor of their severity[4,6].

In a recent meta-analysis enclosing 33 cohort studies[31], CD smoker patients showed increased risks of disease activity flares [odds ratio (OR) = 1.97; 95%CI: 1.21-2.01], post-surgical flares (OR = 1.97; 95%CI: 1.36-2.85), need for both first surgery (OR = 1.68; 95%CI: 1.33-2.12) and second surgery (OR = 2.17; 95%CI: 1.63-2.89). Conversely, the risk of such events was significantly reduced by smoking discontinuation[31-33]. Moreover, smoking has been reported as a well-established risk factor in HS by the European S1 guidelines for the treatment of HS/acne inversa[34]. An association between prevalence of HS and current smoking was found in a French cohort comprising about 10000 subjects (OR = 12.55; 95%CI: 8.58-18.38). This association was not demonstrated in former smokers[35]. Despite this evidence, some aspects of the correlation between HS severity and smoking remain controversial. Indeed, Sartorius *et al*[36] demonstrated a more severe course in active smokers as compared to non-smokers (P = 0.03), even though no statistical difference with former smokers was observed. Conversely, no effect of smoking on disease severity was found in a cohort study enclosing 268 HS patients[37].

Although the relationship between smoking and both diseases is supported by evidence, a hypothetical shared pathogenetic mechanism remains unclear and may be different for HS and CD. Indeed, nicotine may act in HS by multiple pathways, *i.e.,* over-stimulation of the sweat gland with a possible duct obstruction and consequent inflammation, chemotaxis for neutrophils, over-expression of tumor necrosis factor (TNF) alpha in keratinocytes and thickening of epidermidis by means of non-neuronal acetylcholine[38]. Simultaneously, in CD nicotine determines a more aggressive disease pattern, probably causing ischemia of microvessels, due to the implementation of carbon monoxide concentration, and by decreasing the expression of anti-inflammatory cytokines[37]. Finally, smoking cessation improves CD course, however this topic has not been largely investigated in HS[36].

On the other hand, it is well known that smoking does not affect UC course. In detail, nicotine may modulate the immune system by means of its binding to nicotine acetylcholine receptor α7 subunit expressed on macrophage, leading to a reduction of TNF-alpha and inflammation[39].

In conclusion, even if smoking represents a crucial pathogenic factor for both CD and HS, there is currently a lack of studies analyzing a possible shared pathway.

Genetic susceptibility

A role for inheritance in HS and CD pathogenesis has been supposed. Up to 40% of patients with HS show a familial history and an autosomal dominant pattern of inheritance has been observed in some familial cases[6]. Two loci on chromosome 6 and 19, and another one on chromosome 1 (1p21.1-1q25.3) have been linked to HS[6,40,41]. However, a recent report by Al-Ali *et al*[42] did not report any association between the locus 1p21.1-1q25.3 and this disease. Additionally, mutations involving presenilin-1 (PSEN1), presenilin enhancer-2 (PSENEN) and nicastrin (NCSTN) genes, which determine the inactivation of the gamma-secretase enzyme complex, have also been related to HS. The mutation of this enzyme complex is involved in HS pathogenesis via aberrant trichilemmal keratinization[6,41-44].

As for CD, the nucleotide-binding oligomerization domain containing 2 (*NOD2*) gene has been described as a possible inherited factor. Three different mutations have been identified in Caucasian CD patients: one frameshift and two missense mutations[45,46]. This gene is involved in intestinal homeostasis by detecting peptidoglycan released from the gut microbiota and driving a nuclear factor-κB (NF-κB)-mediated inflammatory response. The alteration of this process is supposed to play a role in the development of chronic intestinal inflammation[46].

A recent study by Janse *et al*[26] tried to identify a genetic link between HS and CD. The authors evaluated three different genes, *i.e.,* ELOVL fatty acid elongase 7 (*ELOVL7*) gene on chromosome 5, sulfotransferase family cytosolic 1B member 1 (*SULT1B1*) and sulfotransferase family 1E member 1(*SULT1E1*) genes on chromosome 4. These genes on chromosome 4 originate from the sulfotransferase family, encoding for enzymes that catalyze the sulphate conjugation of hormones, drugs, neurotransmitters and xenobiotic compounds. *SULT1E1* encodes for an enzyme regulating estrogen homeostasis[47]. These hormones seem to be involved in HS clinical course. Indeed, the reactivation of the disease usually occurs during hypoestrogenic states, thus estrogens seem to play a protective role[48]. Additionally, since adiposity is another supposed risk factor for HS, the expression of SULT1E1 in the abdominal subcutaneous tissue of obese people may be considered further evidence of the role of obesity[6]. Moreover, Ahima *et al*[47] demonstrated the co-expression of estrogen

sulfotransferase and TNF-alpha in abdominal adipose tissue of obese subjects. This last pro-inflammatory cytokine has a role in HS and CD pathogenesis as well as representing a therapeutic target for both diseases[49].

However, further studies are needed to investigate the genetic association between HS and CD.

Microbiota

Although the pathogenesis of IBD and HS is generally linked to alterations of the immune response[4,42], recent findings suggest a role for intestinal and skin microbiota, respectively[50,51].

The frequent finding of *Staphylococcus aureus* (*S. aureus*) and coagulase-negative staphylococci (CoNS) on HS cutaneous lesions suggests a bacterial involvement in disease pathogenesis[49].

Kurzen *et al*[52] supposed that nicotine may stimulate the growth of *S. aureus*. Jemec *et al*[53] suggested that *S. aureus* could induce the initial development process of HS, since it influences a series of anatomical alterations in the hair follicles facilitating inflammation and necrosis.

CoNS, in particular *Staphylococcus epidermidis* (*S. epidermidis*), usually are non-pathogenic microorganisms and commensals of the normal skin flora[54]. Lapins *et al*[55] found CoNS in 21 patients with HS. Sixteen out of the 21 patients showed CoNS in the deep levels of the skin, and in 9 of them CoNS were the only bacteria isolated, thus presuming a promoting activity for these germs in HS inflammation. A histological retrospective study analyzing 27 patients with HS showed the presence of *S. epidermidis*-related biofilm (*i. e.*, an extracellular matrix used by bacteria as a protective cover against host defense mechanisms and antimicrobial agents) in one-fifth of the samples located in hair follicles and sinus tracts[56].

Since microflora varies in the different cutaneous regions of the body, in relation to different distributions of hair follicles and glands, two different profiles of HS patients have been identified in a recent report by Guet-Revillet *et al*[57]. *Staphylococcus lugdunensis* was cultured from 58% of HS lesions, that were almost exclusively Hurley stage 1 lesions and more frequently located on the buttocks and the breasts, whereas a polymicrobial flora (strict anaerobes and/or anaerobic actinomycetes and/or streptococci of the milleri group) was predominantly associated with Hurley stage 2 and stage 3 lesions, especially in the axilla, and inguinal and gluteal folds.

Finally, antibiotics represent a treatment option for HS. In this regard, both topic and oral administrations act by killing involved bacteria and determining an indirect immunomodulation with reduction of pro-inflammatory cytokines and induction of neutrophil apoptosis[6].

With regard to IBD pathogenesis, modification of intestinal microflora, including about 1000 bacterial species, has been proposed as a promoting factor. Moreover, different bacterial compositions affect different sites of digestive system inflammation in animal models[51]. Indeed, in germ IL-10-/-germ free mice, bacterial colonization of *Escheria coli* or *Bilophila wadworthia* led to cecum or distal colon involvement, respectively[58]. Couturier-Maillard *et al*[59] described a potential link between genetic factors and microbiome modulation. They transplanted fecal microbiota from healthy wild-type mice to NOD2 deficient ones, obtaining a reduction of IBD risk. Conversely, disease risk rose in wild-type mice that received fecal microbiota from NOD2-deficient ones.

Smoking, as previously described for HS, could determine microbiota alterations, also in the gut with a reduction of Firmicutes and Actinobacteria and an increase of Proteobacteria and Bacteroides[60,61].

The modulation of gut microbiota is a potential therapeutic target in IBD and antibiotics, such as metronidazole and ciprofloxacin, which are currently used in Crohn's colitis, ileocolitis and pouchitis[3,51]. Nevertheless, tetracyclines, antibiotics largely used for HS, showed a Hazard Ratio for developing IBD, for any exposure to these drugs, of 1.39 (95%CI: 1.02-1.90) even if no clear explanation of the mechanism was found[62]. Additionally, a meta-analysis[63] of 11 observational studies, including 7208 IBD patients, demonstrated an OD of 1.57 (95%CI: 1.27-1.94) for IBD development after the exposure to any antibiotic. This risk was higher for CD (OR = 1.74; 95%CI: 1.35-2.23), metronidazole (OR = 5.01, 95%CI: 1.65-15.25), fluoroquinolones (OR = 1.79, 95%CI: 1.03-3.12) and in children (OR = 2.75; 95%CI: 1.72-4.38). Only the penicillin class was not associated with IBD onset.

THERAPEUTIC ANALOGIES

IBD and HS may show some similarities in the therapeutic management, since they may be controlled by some immunomodulatory drugs.

Indeed, HS may benefit from anti-TNF-alpha biologic therapy, similarly to IBD. Numerous case reports have demonstrated that infliximab improves skin lesions in patients with both CD and HS[18, 20,21,64,65]. On these bases, patients suffering from HS have been treated off-label with infliximab and etanercept, with a remission rate of about 35% and a decrease in HS activity of 50%[49,66]. In a systematic review by Haslund *et al*[67], almost all HS treated patients experienced a positive effect. Infliximab therapy is indicated in moderate-severe HS and is well tolerated, reduces skin pain, decreases disease severity and improves quality of life[49]. However, the long-term results are rather poor. Adalimumab has been recently approved by Food and Drug Administration for HS treatment. This FDA approval is based on the results of two pivotal Phase 3 studies, PIONEER I and PIONEER II[68-70].

Additionally, Ustekinumab is a monoclonal antibody

that selectively targets IL-12 and IL-23, which has been proposed for both IBD and HS treatment. In a setting of 17 HS patients, Ustekinumab allowed, after 40 wk, a moderate improvement in the 82% and a complete clinical response in the 47%[71]. A similar success rate, ranging from 46% to 65%, has been found in patients affected by CD who did not benefit from other anti-TNF alpha biologic agents[72,73].

Finally, other immunomodulators, such as corticosteroids and cyclosporine, have been proven to be effective for HS[73-76], similarly to IBD, thus supporting a possible link. However, the general level of evidence for these drugs is very low, given the small number of HS patients described in the literature so far and the lack of randomized controlled studies.

CONCLUSIVE REMARKS

IBD and HS share a chronic inflammatory trait. Despite an association between these two conditions having been reported only anecdotally, in recent years novel clinical investigations performed on large scale have shed new light on their association. The link between HS and IBD - CD in particular - could be stronger than expected. However, epidemiologic data is not supported by strong basic studies. Despite some evidence having shown that immune dysregulation, alteration of microbiota, genetic factors and tobacco smoking may underlie both diseases[52,59,77], a convincing *in vivo* proof has not yet been found. Additionally, the common therapeutic scenario described for IBD and HS might be another clue for their association.

CONCLUSION

An unregulated inflammation leading to microscopic granulomatous wounds may cause the lesions typical of both diseases, particularly when they coexist. However, this is still a largely unexplored field, and further studies are required to elucidate their pathogenesis and possible therapeutic approaches, as well as the interconnection between the disorders and the consequent practical implications. Indeed, despite the association between HS and IBD having been under-evaluated up to now, our pooled results show that the mean prevalence of HS in IBD is 12.8%, with a peak for CD (17.3%). Therefore, an existent link between these two conditions may be argued. On these bases, a careful skin examination should usually be performed in IBD patients, since the association CD-HS may be very disabling. Therefore, an early detection of HS in IBD could prevent the worsening of the skin disorder, thus avoiding the need ~~of~~ for some toxic medications.

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REVIEW

Current and emerging therapies in unresectable and recurrent gastric cancer

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Abstract

Gastric cancer is one of the most lethal cancers worldwide despite many advances and options in therapy. As it is often diagnosed at an advanced stage, prognosis is poor with a median overall survival of less than twelve months. Chemotherapy remains the mainstay of treatment for these patients but it confers only a moderate survival advantage. There remains a need for new targeted treatment options and a way to better define patient populations who will benefit from these agents. In the past few years, there has been a better understanding of the biology, molecular profiling, and heterogeneity of gastric cancer. Our increased knowledge has led to the identification of gastric cancer subtypes and to the development of new targeted therapeutic agents. There are now two new targeted agents, trastuzumab and ramucirumab, that have recently been approved for the treatment of advanced and metastatic gastric cancer. There are also many other actively investigated targets, including epidermal growth factor receptor, the phosphatadylinositol 3-kinase/protein kinase B/mammalian target of rapamycin pathway, c-Met, poly ADP-ribose polymerase, and immune checkpoint inhibition. In this review, we discuss the current management of advanced gastric cancer as well as emerging targeted therapies and immunotherapy.

Key words: Advanced gastric cancer; Immunotherapy; Human epidermal growth factor receptor type 2; Targeted therapy; Vascular endothelial growth factor receptor

Core tip: Despite many advances in medical and surgical treatments, gastric cancer remains the second leading cause of cancer deaths. There is a greater understanding of the molecular heterogeneity of gastric cancer in recent years, resulting in the development and clinical investigation of different targeted agents. This review will discuss current treatment strategies and highlight targeted therapies and emerging drugs for advanced gastric cancer.

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INTRODUCTION

Gastric cancer is the fourth most common cancer and second leading cause of cancer deaths worldwide[1-3]. Gastric cancer is commonly diagnosed at an advanced stage and those patients with advanced disease have a median survival of less than 1 year[4]. Incidence rates and location of the tumor vary considerably between geographic regions. The highest incidence is in East Asia, Eastern Europe and parts of South and Central America where adenocarcinomas of the distal stomach are more prevalent. Cancers located in the proximal stomach or at the gastroesophageal junction (GEJ) are more prevalent in Western Europe and North America[5].

For patients with locally advanced and metastatic disease, chemotherapy remains the mainstay of treatment. Treatment options include platinums, irinotecan, epirubicin, fluoropyrimidines, and taxanes. The addition of a third drug to a two agent regimen increases the response rate with a modest survival improvement but at the expense of increased toxicity[6].

In recent years, advances in the understanding of the biology and molecular profiling of gastric cancer have led to the development of targeted treatments and to a better survival in select patients with advanced disease. There are now two new targeted agents, trastuzumab and ramucirumab, that have been approved in the last 5 years for the treatment of advanced or metastatic gastric cancer. Many more targeted therapies are currently being actively investigated.

In this review, we discuss the management of advanced gastric cancer and the progress in recent years in targeted therapy and immunotherapy.

CHEMOTHERAPY

Gastric cancer is a chemotherapy-sensitive disease with multiple active agents, including fluoropyrimidines, anthracyclines, platinum agents, taxanes, and irinotecan. Treatment of advanced gastric cancer with chemotherapy confers a moderate survival advantage and is primarily palliative. Combination therapy is associated with a higher response rate and increased survival when compared to single agents. The combination of cisplatin and fluorouracil (CF), or with epirubicin in a triple-drug regimen (ECF), has been the most commonly used doublet and triplet regimens. Newer agents were added to these regimens to try to improve response rate (RR), time to progression (TTP) and overall survival (OS). These trials are listed in Table 1.

The addition of docetaxel to cisplatin and fluorouracil (DCF) was shown to be associated with improvement of RR (37% *vs* 25%, $P = 0.01$), TTP (5.6 mo *vs* 3.7 mo, $P < 0.001$), and OS (9.2 mo *vs* 8.6 mo, $P = 0.02$); however there were significant grade 3 to 4 toxicities, including a high rate of febrile neutropenia[7]. These toxicities limited the adoption of this regimen into clinical practice.

Oxaliplatin (O) and oral fluoropyrimidines - capecitabine (X) and S-1 - have been substituted for cisplatin and fluorouracil (5-FU) respectively, and found to be noninferior and less toxic[8-10]. The phase Ⅲ REAL-2 study evaluated the efficacy of oxaliplatin and capecitabine in a 2 × 2 noninferiority trial with four regimens: ECF (control arm), ECX, EOF, and EOX. The median survival times were 9.9 mo, 9.9 mo, 9.3 mo and 11.2 mo respectively[9]. Progression free survival (PFS) and RR did not differ significantly between the different regimens. This study has led to the widespread use of oxaliplatin-based regimens in the frontline treatment of advanced gastric and GEJ cancer.

In Japan, the SPIRITS trial showed that the combination of cisplatin and S-1 (CS) significantly improved OS when compared to S-1 monotherapy (13 mo *vs* 11 mo), leading to this doublet being considered standard first-line in Japan[11]. However, in the United States and Europe, the FLAGS study showed no improvement in outcome when substituting S-1 for 5-FU in combination with cisplatin, so S-1 remains unlicensed in these areas[12].

Irinotecan has also been evaluated in combination with fluorouracil in patients with advanced gastric cancer with no significant differences in response rate, progression free and overall survival compared to the standard care[13,14]. This regimen was found to be less toxic so irinotecan has now been incorporated into the treatment approach.

Although most patients receive first-line chemotherapy, patients who progress after treatment usually have a worsened performance status, which limits treatment options. However, recent studies assessed the administration of irinotecan or docetaxel monotherapy as second-line therapy compared to best supportive care and demonstrated a survival advantage with chemotherapy[15-17]. Therefore, it is now considered standard of care for appropriate patients with a preserved performance status to receive second-line chemotherapy although no standard regimen has been established. A recent trial reported that irinotecan and taxanes have similar survival outcomes[18].

Despite all these treatments, however, the median survival is less than 1 year. There remains a need for new treatment options with targeted therapy and a way to identify which patients would benefit from these new agents.

Table 1 First line chemotherapy completed trials

Ref.	Arms	*n*	TTP/PFS (mo)	OS (mo)
Van Cutsem *et al*[7]	DCF *vs* CF	445	TTP: 5.6 *vs* 3.7 *P* < 0.001	9.2 *vs* 8.6 *P* = 0.02
Al-Batran *et al*[8]	FLO *vs* FLP	220	PFS: 5.8 *vs* 3.9 *P* = 0.077	10.7 *vs* 8.8
REAL-2[9]	ECF *vs* ECX *vs* EOF *vs* EOX	1002	PFS: 6.2 *vs* 6.7 *vs* 6.5 *vs* 7.0	9.9 *vs* 9.9 *vs* 9.3 *vs* 11.2
Kang *et al*[10]	XP *vs* FP	316	PFS: 5.6 *vs* 5.0	10.5 *vs* 9.3
SPIRITS[11]	CS *vs* S-1	298	PFS: 6.0 *vs* 4.0 *P* < 0.0001	13.0 *vs* 11.0 *P* = 0.04
FLAGS[12]	CS *vs* CF	1053	PFS: 4.8 *vs* 5.5 *P* = 0.920	8.6 *vs* 7.9 *P* = 0.20
Dank *et al*[13]	IF *vs* CF	333	TTP: 5.0 *vs* 4.2 *P* = 0.088	9.0 *vs* 8.7
Guimbaud *et al*[14]	FOLFIRI *vs* ECX	416	PFS: 5.3 *vs* 5.8 *P* = 0.960	9.5 *vs* 9.7 *P* = 0.95

DCF: Docetaxel/cisplatin/fluorouracil; CF: Cisplatin/fluorouracil; FLO: Fluorouracil/leucovorin/oxaliplatin; FLP: Fluorouracil/leucovorin/cisplatin; ECF: Epirubicin/cisplatin/fluorouracil; ECX: Epirubicin/cisplatin/capecitabine; EOF: Epirubicin/oxaliplatin/fluorouracil; EOX: Epirubicin/oxaliplatin/capecitabine; XP: Cisplatin/capecitabine; FP: Cisplatin/fluorouracil; CS: Cisplatin/S-1; SOX: S-1/oxaliplatin; IF: Irinotecan/fluorouracil; FOLFIRI: Fluorouracil/leucovorin/irinotecan.

MOLECULAR CLASSIFICATION

Gastric cancer is a heterogeneous disease; however, it wasn't until recently that we developed a better understanding of the molecular and genomic basis of gastric cancer. The Cancer Genome Atlas proposed four molecularly unique subtypes of gastric cancer: tumors positive for Epstein-Barr virus (EBV), microsatellite unstable (MSI) tumors, genomically stable tumors and tumors with chromosomal instability[19].

Tumors associated with EBV were predominantly in the fundus or body and were shown to have a higher prevalence of mutations in *PIK3CA* (approximately 80%), extensive DNA hypermethylation, overexpression of PD-L1 and PD-L2, and EBV-CpG island methylator phenotype (CIMP) expression. MSI tumors were diagnosed at a relatively older age (median age 72 years) and showed elevated mutation rates, gastric CIMP and *MLH1* silencing but generally lacked targetable amplifications. Unlike in colorectal cancer, BRAF mutations were not seen in gastric MSI tumors. Genomically stable tumors tended to be diagnosed at an earlier age (median age 59 years) and were enriched for diffuse histology, associated with *CDH1* and *RHOA* mutations and CLDN18-ARHGAP fusion, which is implicated in cell motility. Almost half of gastric tumors demonstrated chromosomal instability, which was predominantly intestinal histology with an elevated frequency in the GEJ and cardia and showed marked aneuploidy. They were associated with *TP53* mutation with RTK-RAS activation.

This study showed distinct genomic features in the different molecular subtypes that provide a guide to targeted therapy and allow for development of clinical trials to explore therapies in defined sets of patients.

TARGETED THERAPIES

Human epidermal growth factor receptor 2 inhibitors

Human epidermal growth factor receptor 2 (HER2) is a transmembrane tyrosine kinase receptor belonging to the epidermal growth factor receptor (EGFR) family. Activation of the HER2 receptor activates downstream signals in the Ras/Raf/mitogen-activated protein kinase (MAPK) and phosphatadylinositol 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) pathways that are responsible for regulating a variety of tumor biology, such as cell growth, differentiation, and survival[20-22]. The reported HER2 positivity in patients with gastric cancer ranges widely from 6% to 34% depending on the histologic subtype and location with the highest rates of expression observed in intestinal type tumors and in cancers located in the GEJ[23-25]. Unlike in breast cancer where overexpression of HER2 associates with a more aggressive tumor[26], the prognostic role of HER2 in gastric cancer is less clear. Also HER2 testing in gastric cancer differs from that in breast cancer because of inherent differences in tumor biology - gastric cancer more frequently shows tumor heterogeneity and incomplete membrane staining due to its high frequency of glandular formation[27]. There are several different strategies for targeting HER2: anti-HER2 monoclonal antibodies or small-molecule tyrosine kinase inhibitors (TKIs). Table 2 summarizes the completed trials with targeted agents in advanced gastric cancer and Table 3 outlines the ongoing trials.

The first targeted agent approved in gastric cancer was trastuzumab, which acts on the extracellular domain of the HER2 receptor and inhibits HER2-mediated signaling. Trastuzumab for gastric cancer (ToGA) was a phase Ⅲ, randomized controlled trial

Table 2 Targeted therapy completed trials

Ref.	Name Phase	Indication	Line	Arms	*n*	PFS (mo)	OS (mo)
HER2							
[28]	ToGA Phase Ⅲ	HER2(+) Adv/Met GC and GEJ	1st	Fluoropyrimidine/cisplatin ± trastuzumab	594	6.7 *vs* 5.5 *P* = 0.0002	13.8 *vs* 11.1 *P* = 0.0050
[29]	LOGiC Phase Ⅲ	HER2(+) Adv/Met GC and GEJ	1st	CapeOx ± lapatinib	545	6.4 *vs* 5.4 p = 0.1000	12.2 *vs* 10.5 *P* = 0.3492
[30]	TyTAN Phase Ⅲ	HER2(+) Adv/Met GC and GEJ	2nd	Paclitaxel ± lapatinib	261	5.4 *vs* 4.4 *P* = 0.2441	11.0 *vs* 8.9 *P* = 0.2088
EGFR							
[36]	EXPAND Phase Ⅲ	Adv/Met GC and GEJ	1st	Capecitabine/cisplatin ± cetuximab	904	4.4 *vs* 5.9 *P* = 0.3200	9.4 *vs* 10.7 *P* = 0.9500
[37]	REAL3 Phase Ⅲ	Adv/Met GC and GEJ	1st	EOX *vs* modified EOX + panitumumab	553	6.0 *vs* 7.4 *P* = 0.0680	8.8 *vs* 11.3 *P* = 0.013
VEGFR							
[48]	Sunitinib Phase Ⅱ	Adv GC and GEJ	2nd	Sunitinib	78	2.3	6.8
[49]	Sunitinib Phase Ⅱ	Adv GC and GEJ	2nd or 3rd	FOLFIRI ± sunitinib	91	3.6 *vs* 3.3 *P* = 0.6600	10.5 *vs* 9.0 *P* = 0.2100
[50]	Sorafenib Phase Ⅱ	Adv/Met GC and GEJ	1st	Docetaxel/cisplatin + sorafenib	44	5.8	13.6
[51]	Sorafenib Phase Ⅱ	Adv GC and GEJ	2nd	Oxaliplatin + sorafenib	40	3	6.5
[53]	Regorafenib Phase Ⅱ	Adv GC and GEJ	2nd or 3rd	Regorafenib *vs* placebo	152	11.1 wk *vs* 3.9 wk *P* < 0.0001	25 wk *vs* 19.4 wk *P* = 0.1100
[41]	AVAGAST Phase Ⅲ	Adv GC and GEJ	1st	Capecitabine/cisplatin ± bevacizumab	774	6.7 *vs* 5.3 *P* = 0.0037	12.1 *vs* 10.1 *P* = 0.1002
[43]	REGARD Phase Ⅲ	Met GC and GEJ	2nd	BSC ± ramucirumab	355	2.1 *vs* 1.3 *P* < 0.0001	5.2 *vs* 3.8 *P* = 0.0473
[44,45]	RAINBOW Phase Ⅲ	Met GC and GEJ	2nd	Paclitaxel ± ramucirumab	665	4.4 *vs* 2.86 *P* < 0.0001	9.63 *vs* 7.36 *P* = 0.0169
[47]	Apatinib Phase Ⅲ	Adv GC and GEJ	3rd	Apatinib *vs* placebo	270	78 d *vs* 53 d *P* < 0.0001	195 d *vs* 140 d *P* < 0.016
mTOR							
[58]	GRANITE-1 Phase Ⅲ	Adv GC and GEJ	2nd or 3rd	BSC ± everolimus	656	1.7 *vs* 1.4 *P* = 0.0010	5.4 *vs* 4.3 *P* = 0.124

Adv: Advanced; Met: Metastatic; GC: Gastric cancer; GEJ: Gastroesophageal junction; CapeOx: Capecitabine/oxaliplatin; EOX: Epirubicin/oxaliplatin/capecitabine; FOLFIRI: Fluorouracil/leucovorin/irinotecan; BSC: Best supportive care; HER2: Human epidermal growth factor receptor 2; EGFR: Epidermal growth factor receptor; VEGFR: Vascular endothelial growth factor receptor; mTOR: Mammalian target of rapamyin.

involving 584 treatment naïve patients with metastatic or locally advanced unresectable HER2-overexpressing (defined as IHC3+ or FISH positive) gastric or GEJ adenocarcinoma[28]. The addition of trastuzumab to standard chemotherapy demonstrated a significant clinical benefit with higher response rate (47% *vs* 35%), improved progression-free survival (PFS) (6.7 mo *vs* 5.5 mo) and improved OS (13.8 mo *vs* 11.1 mo) compared to the chemotherapy alone arm. In an exploratory analysis, trastuzumab had the greatest survival benefit in patients with IHC3+ tumors, and with FISH+/IHC2+ tumors and ineffective in those with FISH positive but IHC 0 or 1+ tumors. Based on this data, trastuzumab was approved in combination with chemotherapy for the treatment of patients with metastatic HER2-overexpressing gastric or GEJ adenocarcinoma who have not received prior treatment. The ongoing HELOISE trial is evaluating whether a higher dose of trastuzumab in patients with a high tumor burden will have improved OS compared to the standard dosing [NCT01450696].

Given these significant results from the ToGA study, other strategies to target HER2 have been evaluated. Lapatinib is a tyrosine kinase inhibitor of EGFR and HER2 that binds to the intracellular ATP binding site of these kinases and interferes with their activation. However, unlike with trastuzumab, the trials with lapatinib failed to meet their primary endpoints. The phase Ⅲ LOGiC trial evaluated the addition of lapatinib to capecitabine and oxaliplatin as first line therapy in 545 patients with HER2 positive advanced gastric and GEJ adenocarcinomas[29]. Median OS was 12.2 *vs* 10.5 mo in the lapatinib arm compared to the placebo arm with a hazard ratio (HR) of 0.91 (95%CI: 0.73-1.12, *P* = 0.35). However, subgroup analysis showed that certain subgroups - Asian patients (median OS 16.5 mo *vs* 10.9 mo, HR = 0.91) and those under 60 years (median OS 12.9 mo *vs* 9 mo, HR = 0.69) - had significant improvements in OS. Similar negative results were seen in the second line setting: the TyTAN trial compared weekly paclitaxel with or without lapatinib and although the median OS was prolonged by two months (11.0 mo *vs* 8.9 mo, HR = 0.84), it was not statistically significant[30]. The subgroup of

Table 3 Ongoing trials

Name Phase	Indication	Line	Agent	ClinicalTrials.gov Identifier
HER2				
HELOISE Phase Ⅲ	HER2(+) Met GC and GEJ	1st	Trastuzumab	NCT01450696
JACOB Phase Ⅲ	HER2(+) Met GC and GEJ	1st	Pertuzumab	NCT01774786
VEGFR				
RAINFALL Phase Ⅲ	HER2(-) Met GC and GEJ	1st	Ramucirumab	NCT02314117
PARP				
Olaparib Phase Ⅲ	Adv GC and GEJ	2nd	Olaparib	NCT01924533
Immune checkpoints				
KEYNOTE-059 Phase Ⅱ	Adv GC and GEJ		Pembrolizumab	NCT02335411
KEYNOTE-061 Phase Ⅲ	Adv GC and GEJ	2nd	Pembrolizumab	NCT02370498
KEYNOTE-062 Phase Ⅲ	Adv GC and GEJ	1st	Pembrolizumab	NCT02494583
MEDI4736 Phase Ⅰ/Ⅱ	Advanced solid tumors		MEDI4736	NCT01693562
JAVELIN Gastric 100 Phase Ⅲ	Adv/Met GC and GEJ	1st	Avelumab	NCT02625610
JAVELIN Gastric 300 Phase Ⅲ	Met/recurrent GC and GEJ	3rd	Avelumab	NCT02625623
Phase Ⅰ/Ⅱ	Met/recurrent GC and GEJ		MEDI4736 + Tremelimumab *vs* MEDI4736 *vs* Tremelimumab	NCT02340975
Phase Ⅰ/Ⅱ	Advanced solid tumors		Nivolumab +/- Ipilimumab	NCT01928394

Adv: Advanced; Met: Metastatic; GC: Gastric cancer; GEJ: Gastroesophageal junction; HER2: Human epidermal growth factor receptor 2; VEGFR: Vascular endothelial growth factor receptor; PARP: Poly ADP-ribose polymerase.

patients with IHC3+, however, did have a significant benefit in both PFS (5.6 mo *vs* 4.2 mo) and OS (14 mo *vs* 7.6 mo).

Two other drugs that have been FDA approved for the treatment of patients with metastatic HER2 positive breast cancer are being investigated in HER2 positive gastric cancer. Pertuzumab is an antibody that binds to a different site on HER2 than trastuzumab and inhibits the dimerization of HER2. The phase Ⅲ JACOB trial will evaluate the efficacy and safety of pertuzumab in combination with trastuzumab, fluoropyrimidine and cisplatin [NCT01774786]. TDM-1 is an antibody-drug conjugate of trastuzumab and a potent microtubule inhibitor DM1. The multicenter phase Ⅱ/Ⅲ GATSBY trial to evaluate TDM-1 *vs* a taxane in advanced gastric cancer as second line did not show an efficacy benefit of TDM-1 over taxane[31].

EGFR inhibitors

EGFR (HER1) is a member of the same family of tyrosine kinase receptors as HER2 and it activates the same intracellular signaling pathways that are responsible for regulating cell growth, differentiation, and survival[32,33]. EGFR overexpression occurs in 30%-60% of gastric cancer and is associated with a worse prognosis[34,35]. However, studies evaluating antibody inhibitors of EGFR have failed to demonstrate a survival advantage.

Cetuximab is a chimeric monoclonal IgG1 antibody that binds to the extracellular domain of EGFR and competitively inhibits the binding of EGF and other ligands. The phase Ⅲ trial EXPAND randomized 904 patients to capecitabine and cisplatin with or without cetuximab and did not find progression free or overall survival benefit for the cetuximab group (4.4 mo *vs* 5.6 mo and 9.4 mo *vs* 10.7 mo, respectively)[36]. Response rates were comparable between the two arms (30% *vs* 29%) but the cetuximab arm resulted in a higher rate of grade 3 and 4 toxicity (88% *vs* 77%).

Panitumumab is a fully humanized monoclonal IgG2 antibody targeting EGFR. The phase Ⅱ/Ⅲ REAL3 trial evaluated the efficacy of epirubicin, oxaliplatin, and capecitabine with or without panitumumab as first line therapy[37]. The phase Ⅲ study did not show any benefit and actually showed a lower survival in the experimental arm at a preplanned interim analysis (median OS 8.8 mo *vs* 11.3 mo) so it was discontinued prematurely.

Vascular endothelial growth factor receptor inhibitors

Pathological angiogenesis is crucial for tumor growth, survival and metastases. VEGF is an important regulator of angiogenesis and acts on its vascular endothelial growth factor receptor (VEGFR) to stimulate endothelial cells to divide and migrate to form new blood vessels or sprout from existing ones and to help newly formed

blood vessels survive[38]. VEGFR is overexpressed in 30%-60% of gastric cancer and is a predictor of poor prognosis[39,40]. Trials evaluating anti-VEGF agents are listed in Table 2.

Bevacizumab is a recombinant humanized IgG1 monoclonal antibody against VEGF. AVAGAST was a large randomized phase Ⅲ study evaluating the addition of bevacizumab to capecitabine and cisplatin[41]. Median PFS (6.7 mo *vs* 5.3 mo) and overall response rate (ORR) (46% *vs* 37.4%) was significantly improved in the bevacizumab arm but the primary endpoint of OS was not met (12.1 mo *vs* 10.1 mo, $P = 0.1002$). In subgroup analysis, patients from North and South America showed survival benefit from the addition of bevacizumab (11.5 mo *vs* 6.8 mo, HR = 0.63, 95%CI: 0.43-0.94), patients from Europe showed a trend toward benefit (HR = 0.85, 95%CI: 0.63-1.14), and patients from Asia had no benefit (HR = 0.97, 95%CI: 0.75-1.25), further suggesting heterogeneity of this disease worldwide. Similar negative results were seen in the AVATAR study where bevacizumab was added to capecitabine and cisplatin in Asian patients with advanced gastric cancer[42].

Ramucirumab is a fully humanized monoclonal antibody against VEGFR-2. The phase Ⅲ REGARD trial compared ramucirumab monotherapy with best supportive care in the second line[43]. The study showed improved median PFS (2.1 mo *vs* 1.3 mo, $P < 0.001$) and median OS (5.2 mo *vs* 3.8 mo, $P = 0.047$). In the phase Ⅲ RAINBOW study, advanced gastric or GEJ adenocarcinoma patients were randomized to paclitaxel with or without ramucirumab in the second line setting[44,45]. The addition of ramucirumab showed improved OS of 9.6 mo *vs* 7.4 mo compared to paclitaxel alone ($P = 0.0169$) and improved PFS (4.4 mo *vs* 2.9 mo). Based on these trial results, ramucirumab was approved as a single agent for treatment of patients with advanced gastric or GEJ cancer after progressing on prior treatment, as well as in combination with paclitaxel. This is the first approval of a biologic agent in an unselected population with gastric and GEJ cancers. Ramucirumab was also tested in the first line setting in combination with FOLFOX, but it did not show an improvement in the primary endpoint of PFS or median[46]. It is also being studied in the phase Ⅲ RAINFALL trial comparing PFS in patients with HER2-negative, metastatic gastric or GEJ adenocarcinoma receiving ramucirumab with cisplatin and fluoropyrimidine *vs* cisplatin and fluoropyrimidine as first line treatment [NCT02314117].

A phase Ⅲ trial assessing a TKI against VEGFR, apatinib, with a two-to-one randomization to apatinib *vs* placebo in the third line setting in advanced gastric cancer showed that median OS was significantly prolonged in the apatinib group of 195 d *vs* 140 d ($P < 0.016$), as was median PFS of 78 d *vs* 53 d ($P < 0.0001$)[47].

Sunitinib and sorafenib are multitargeted TKIs that inhibit VEGFR as well as other kinases. Phase Ⅱ trials have been conducted both as monotherapy and in combination with chemotherapy and have shown mixed results[48-51]. These results are summarized in Table 2. Pazopanib, another multitargeted TKI that inhibits angiogenesis, showed marginal efficacy in a phase Ⅱ trial as first line with 5-FU/oxaliplatin[52]. Data on a phase Ⅱ trial of regorafenib, a multi-kinase inhibitor, following progression after 1st or 2nd line chemotherapy demonstrated significantly improved PFS in the regorafenib arm[53]. Pre-specified analyses found the effect of regorafenib to be greater in South Korea than in Australia, New Zealand and Canada.

mTOR inhibitors

PI3K/Akt/mTOR pathway is a major downstream cascade of tyrosine kinase signaling and one of the most frequently altered pathways in malignancies. mTOR, an intracellular key serine/threonine protein kinase, regulates cell growth, motility, cellular metabolism and angiogenesis[54,55]. Dysregulation of this pathway is associated with poor survival and may contribute to resistance to chemotherapy[56,57].

Everolimus, an oral mTOR inhibitor, was evaluated in the phase Ⅲ GRANITE-1 trial where it was compared to best supportive care in advanced gastric cancer that progressed after previous chemotherapy[58]. The trial randomly assigned 656 patients in a 2:1 ratio to everolimus or placebo. Although median PFS was improved (1.68 mo *vs* 1.41 mo, $P < 0.001$), the trial did not meet its primary endpoint of improved OS (5.39 *vs* 4.3 mo, $P = 0.124$). Everolimus is currently being evaluated in combination with paclitaxel as second line treatment in a phase Ⅲ trial [NCT01248403].

c-MET inhibitors

MET is a tyrosine kinase receptor and signals through RAS-MAPK and PI3K-AKT pathways to mediate cell migration, survival, invasion and angiogenesis. Aberrant HGF/MET signaling triggers multiple intracellular signals that lead to tumor growth, proliferation and metastasis[59]. In addition to oncogenesis, aberrant MET signaling has been associated with *in vitro* resistance to cytotoxic agents[60]. c-MET amplification is associated with a higher tumor stage, a more aggressive phenotype and a significantly diminished survival[61,62].

Crizotinib is a small molecule inhibitor of anaplastic lymphoma kinase and MET tyrosine kinase that is approved in non-small cell lung cancer. In a study of patients with gastroesophageal cancer, of the 489 tumors screened, 10 patients (2%) harbored MET amplification (> 5 copies)[63]. These tumors were more likely to be high-grade and present at advanced stages. Two out of these four patients had a clinical response with a delay in tumor progression. However the responses were transient and time to progression in these two patients was 3.7 mo and 3.5 mo.

Rilotumumab is a fully humanized monoclonal IgG2

against HGF that inhibits the binding of HGF to the MET receptor. A phase Ⅱ trial evaluating rilotumumab in combination with ECX in patients with untreated advanced gastroesophageal cancer showed minimally improved median PFS and median OS but an exploratory analysis showed that patients with high MET expression appeared to experience marked clinical benefit from addition of rilotumumab to ECX with improvement in median OS from 5.7 to 11.1 mo (HR = 0.29)[64,65]. These results led to 2 phase Ⅲ studies: RILOMET-1 (rilotumumab in combination with ECX as first-line treatment for advanced MET-positive gastroesophageal cancer) and RILOMET-2 (rilotumumab with cisplatin and capecitabine as first-line therapy in gastric cancer). However, data from RILOMET-1 showed that OS, PFS and ORR were statistically worse in the rilotumumab arm[66]. No subgroups seemed to benefit with rilotumumab, including those with higher percentages of cells with ⩾ 1+ MET expression.

Onartuzumab is a monovalent humanized monoclonal antibody against the MET receptor and prevents HGF binding to MET. Onartuzumab in combination with mFOLFOX6 was evaluated in patients with untreated metastatic gastroesophageal cancer that were HER2-negative and MET-positive (Ⅲ 50% of tumor with moderate-strong intensity staining by IHC based on central review) in a phase Ⅲ trial, MetGastric. The trial showed that the addition of onartuzumab to mFOLFOX6 did not improve PFS in the unselected population or in the MET-positive subgroup[67,68]. Monoclonal antibodies that target c-MET have limited activity as seen in the phase Ⅲ trials with rilotumumab and onartuzumab, and better biomarkers are needed to select patients for trials with c-MET inhibitors.

Poly ADP-ribose polymerase inhibitors

Poly ADP-ribose polymerase (PARP) is a family of proteins that are critical for the function of base excision repair (BER). BER repairs single strand DNA breaks. If these single strand breaks are not repaired, they become double strand breaks which leads to cell death[69]. PARP inhibitors interfere with BER and prevent this repair mechanism which may ultimately lead to death of tumor cells[70].

Olaparib was studied in a second line phase Ⅱ trial for metastatic or recurrent gastric cancer in combination with paclitaxel *vs* paclitaxel alone[71]. The trial found a statistically significant improvement in OS, but not PFS. Initial preclinical data suggested that responsiveness of gastric cancer cell lines to olaparib was associated with low ataxia telangiectasia mutated (ATM) protein levels, so the study performed a subset analysis and found that patients with low ATM showed a larger improvement in OS with olaparib. These results led to a phase Ⅲ trial of olaparib in combination with paclitaxel compared with paclitaxel monotherapy in patients with advanced gastric cancer who have progressed following first line therapy [NCT01924533]. Another PARP inhibitor, veliparib, is currently being studied in a phase I trial with FOLFIRI in patients with advanced gastric cancer [NCT01123876].

IMMUNOTHERAPY

Tumor cells have developed mechanisms in which they modulate the immune system, allowing them to escape the immune cells and provide a shield for which the tumor is able to invade, migrate, and grow[72,73]. Immune checkpoints are inhibitory pathways that maintain self-tolerance and protect tissues from damage when the immune system is active. The expression of immune-checkpoint proteins can be dysregulated by tumors, making this an important immune resistance mechanism[74]. This has led to increasing interest in immunotherapy as a treatment option in multiple solid malignancies.

Cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) was the first immune checkpoint receptor to be clinically targeted. It is expressed exclusively on T cells and is a negative regulator of T-cell activation. There are two fully humanized CTLA4 antibodies, ipilimumab and tremelimumab. After ipilimumab became the first therapy to improve overall survival in patients with advanced melanoma[75], it is now being evaluated in other advanced cancers including gastric cancer. A phase Ⅱ trial to evaluate the efficacy of ipilimumab after first-line chemotherapy in the treatment of unresectable or metastatic gastric or GEJ adenocarcinomas was just completed and awaiting results [NCT01585987]. Tremelimumab was investigated in a phase Ⅱ trial as second-line treatment for patients with metastatic gastric cancer but had an ORR of only 5% and median OS similar to that of second-line chemotherapy[76].

Another immune-checkpoint protein which is expressed on T cells, programmed cell death protein 1 (PD-1), inhibits the activity of the T cell when bound to its ligands PD-L1 and PD-L2 on the surface of a cell. Tumor cells often overexpress PD-L1 or PD-L2 resulting in T cell anergy and escape from immunosurveillance. The KEYNOTE-012 phase Ib study of pembrolizumab, a monoclonal antibody that blocks PD-1 interaction with its ligands, in patients with recurrent and metastatic gastric cancer with PD-L1 tumor positivity based on a prototype IHC assay showed an ORR of 31.6% in Asia Pacific and 30% in the rest of world[77]. These results present an exciting novel strategy in the treatment of advanced gastric cancer. The phase Ⅱ KEYNOTE-059 of pembrolizumab with cisplatin and 5-FU as first-line is currently enrolling [NCT02335411]. Also, two phase Ⅲ trials, KEYNOTE-061 [NCT02370498] of pembrolizumab *vs* paclitaxel as second line therapy and KEYNOTE-062 [NCT02494583] of pembrolizumab alone or in combination with cisplatin and fluoropyrimidine *vs* chemotherapy as first line therapy, are currently ongoing.

There was preliminary evidence of an association

between PD-L1 expression and PFS (P = 0.032) and ORR (P = 0.071) in the KEYNOTE-012 study. This relationship between PD-L1 expression and clinical outcomes was further explored and found that PD-L1 expression level was associated with ORR (1-sided P = 0.10) and ORR was 22% (95%CI: 10-39) by central review and 33% (95%CI: 19-50) by investigator review[78]. The 6-mo PFS rate was 24% and the 6-mo OS rate was 69%. Another biomarker that may be predictive of anti-PD-1 therapy is mismatch repair-deficiency. A phase Ⅱ trial of pembrolizumab for the treatment of colorectal and other GI tumors, including gastric cancer, with mismatch repair-deficiency, or high microsatellite instability (MSI-H), demonstrated high ORR and prolonged PFS [immune-related ORR 71% (5 of 7 patients); immune-related PFS 67% (4 of 6 patients)] when treated with pembrolizumab, supporting the hypothesis that mismatch repair-deficient tumors are more responsive to PD-1 blockade than are mismatch repair-proficient tumors[79].

MEDI4736, a PD-L1 IgG1 antibody, is being studied in a phase Ⅰ/Ⅱ trial in patients with advanced solid tumors including gastric cancer and the results of the phase Ⅰ showed good clinical activity with tumor shrinkage and durable responses[80]. Expansion in multiple cancers is ongoing [NCT01693562]. Another PD-L1 inhibitor, Avelumab (MSB0010718C), is being investigated in phase I trials in advanced cancers [NCT01943461, NCT01772004] and in phase Ⅲ trials in the first line [NCT02625610] and third line [NCT02625623] settings. The combination of CTLA-4 and PDL-1 inhibitors is also being evaluated [NCT01975831, NCT02340975, NCT01928394]. These ongoing trials are outlined in Table 3.

CONCLUSION

Chemotherapy has long been the standard treatment for advanced gastric cancer. Current combination cytotoxic regimens are associated with response rates of ⩾ 40% but median survival is still less than one year. To improve on this outcome, we have made many advances in our knowledge of the molecular etiology and heterogeneity of gastric cancer, which has led to the development of different targeted therapies. The ToGA trial has established trastuzumab as a new standard of care for patients with HER2 positive (IHC3+ or IHC2+/FISH positive) advanced or metastatic gastric cancer, but this benefit is limited to only approximately 20% of patients with advanced disease. Ramucirumab has also been approved recently for treatment in the second line setting and offers a valuable alternative or addition to chemotherapy. Despite these advances, standard therapy for advanced gastric cancer in the first line setting for patients who are not HER2 positive is still combination chemotherapy with either a doublet or triplet of a platinum and a fluoropyrimidine. Second line options are still limited and include irinotecan, docetaxel, paclitaxel with or without ramucirumab or ramucirumab monotherapy.

There remains a need to better define patient populations who will benefit from targeted therapy and predict the response to drugs. The Cancer Genome Atlas Research Network has recently classified four molecular subtypes of gastric cancer and identified other possible targets for future clinical research. This will allow for the development of clinical trials in the future to explore therapies in defined sets of patients. Better use of biomarkers to select these sets of patients to improve outcomes will be crucial. Also, as our understanding of the complex interplay between the tumor, the tumor microenvironment and the immune system expand, use of immunotherapy will continue to grow. Although the optimal use of these agents is not yet defined, they may provide an unmet need for patients who did not benefit or unable to tolerate traditional chemotherapy. Further work is necessary to determine the role of targeted therapy and the combination of targeted agents with cytotoxic agents that will translate into improved survival but the future looks optimistic.

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REVIEW

Multiplex qPCR for serodetection and serotyping of hepatitis viruses: A brief review

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Abstract

The present review describes the current status of multiplex quantitative real time polymerase chain reaction (qPCR) assays developed and used globally for detection and subtyping of hepatitis viruses in body fluids. Several studies have reported the use of multiplex qPCR for the detection of hepatitis viruses, including hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus (HDV), and hepatitis E virus (HEV). In addition, multiplex qPCR has also been developed for genotyping HBV, HCV, and HEV subtypes. Although a single step multiplex qPCR assay for all six hepatitis viruses, *i.e.*, A to G viruses, is not yet reported, it may be available in the near future as the technologies continue to advance. All studies use a conserved region of the viral genome as the basis of amplification and hydrolysis probes as the preferred chemistries for improved detection. Based on a standard plot prepared using varying concentrations of template and the observed threshold cycle value, it is possible to determine the linear dynamic range and to calculate an exact copy number of virus in the specimen. Advantages of multiplex qPCR assay over singleplex or other molecular techniques in samples from patients with co-infection include fast results, low cost, and a single step investigation process.

Key words: Co-infection; Viral genome; Quantitative real-time polymerase chain reaction; Genotyping techniques; Serotyping; Hepatitis viruses

Core tip: The present review describes the worldwide application and the significance of multiplex quantitative real time polymerase chain reaction (qPCR) for simultaneous detection of hepatitis viruses and their subtypes in serum. The published literature has demonstrated that the multiplex qPCR assay is a fast, easy, cost-effective, and sensitive technique for the early diagnosis of hepatitis co-infections. Use of this technique, in comparison to other diagnostic procedures, is increasing in diagnostic laboratories.

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INTRODUCTION

Viral hepatitis is a serious public health problem requiring early diagnosis and timely treatment. There are a number of hepatitis viruses that have already been characterized based on their molecular structure and named alphabetically as hepatitis viruses A, B, C, D, E, and G (HAV, HBV, HCV, HDV, HEV, and HGV), respectively. These are hepatotropic and non-cytopathic in nature and cause liver damage by immune mediated cell lysis[1]. There is an additional group of viruses that cause hepatitis but are not yet characterized. These viruses have been put under the category of non A-G hepatitis viruses. HAV infects mainly the pediatric age group, occurs both sporadically as well as in epidemics, and accounts for an estimated 1.4 million cases annually[2]. Two billion people are suspected to be infected with HBV globally, and approximately 350 million of them suffer from chronic hepatitis B infection[3]. About 25% of adults infected with HBV during childhood are reported to die from hepatocellular carcinoma (HCC) or liver cirrhosis[4]. In addition, 3-4 million people are infected with HCV each year, and a high proportion of them develop chronic HCV infection. A large population infected with HCV dies from serious liver diseases annually[5]. Similarly, reports are also available on HEV infection. In addition to individual viral infection, there are cases of co-infections reported from various parts of the world. Hepatitis A and E infections usually run a benign course of disease and resolve in due course of time without developing chronic diseases. In contrast, hepatitis B and C infections cause severe liver diseases, developing chronicity in a significant number of patients. Interestingly, hepatitis A and E infections in patients with pre-existing HBV or HCV infections lead to the development of serious diseases with a significant rise in morbidity and mortality[6].

The diagnosis of hepatitis viral infections is usually done with serological markers in blood. However, there are situations where serology loses its credibility. For example, serological markers can not differentiate between past and present infections. In addition, serological tests do not address the problem of antigenic variations in viruses, infections with different genotypes, presence of silent carriers, and absence of antibody in early phase of infection[7]. Moreover, the presence of maternal antibodies makes it impossible to detect infections in newborns[8]. In order to have an alternate system, the nucleic acid tests (NAT) based methods were developed for detecting the viral genome in serum for the diagnosis of viral hepatitis. NAT based methods have the benefit of direct examination of the infectious agent's genome in serum[9,10].

The conventional polymerase chain reaction (PCR) is one such NAT based method that has been in practice in some laboratories for the diagnosis of viral hepatitis in the last few years[11]. However, conventional PCR is a lengthy procedure with several technical and operational problems, and so, it is of limited use. In addition, each marker needs to be investigated separately by PCR, and it takes a very long time to reach a final diagnosis. Because of these limitations of conventional PCR, the use of real time PCR was supposed to be a better option for early diagnosis of viral hepatitis in both sporadic and epidemic cases. Real time PCR is one of the latest techniques frequently used for the diagnosis of various infectious diseases, including viral hepatitis. It can detect causative pathogen-related nucleic acid in body fluids in a very short time period. It can also be used to determine different molecular forms and variant molecular species of pathogens, including bacteria, viruses, and several parasites[12,13]. Real time PCR is a specific and sensitive technique and uses specific probes and primers to detect target sequences in the genome. Moreover, this technique is performed on an automated machine without the need of post PCR procedures, thus minimizing cross contamination between samples, simultaneously accelerating the analysis[14].

The recent development of molecular technologies has relayed a strong message to medical researchers to explore ways to further improve the diagnostic procedures. Those researchers working in the area of medical virology have switched from traditional approaches of virus detection in clinical samples to multiplexing for simultaneous detection of multiple pathogens in a single assay[15]. Recently, several PCR based assays coupled with oligonucleotide microarray technology have been designed to allow for the simultaneous detection and genotyping of several viruses, including blood borne pathogens[16], respiratory viruses[17], and adenoviruses[18]. These assays show a significant increase in the sensitivity of detection, reaching 10-100 copies of target RNA/DNA in a sample[19]. Given the ease of performance, short reaction time, low cost, and the ability to monitor the results on a screen, these assays have proved attractive to all diagnostic laboratories furnished with minimal essential facilities. After surveying the literature on the use of PCR based multiplex assays for detecting and genotyping hepatitis viruses, we noticed several attempts to develop multiplex real time PCR assays for hepatitis in the last few years. Here, we provide an up to date review on the development, use, and significance of multiplex qPCR in the field of viral hepatitis.

Table 1 Conserved genomic regions used as templates for amplification of hepatitis viruses in qPCR assays

Virus	Conserved region	Ref.
HAV	5′ UTR	[4,15,20,22]
HBV	S-gene	[4, 19-21]
	X-gene	[15]
HCV	5′ UTR	[4,15,19-21]
HDV	Ribozyme-1	[20]
HEV	ORF2	[15,22]
	ORF3	[20]
HGV	5′ UTR	[20]

HAV: Hepatitis A virus; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HDV: Hepatitis D virus; HEV: Hepatitis E virus; UTR: Untranslated region; ORF: Open reading frame.

EXPERIMENTAL APPROACH FOR MULTIPLEX qPCR

Search for conserved regions

In order to develop a multiplex qPCR assay for multiple pathogens, the first and foremost step is to explore and locate the target region on each pathogen's genome for amplification purpose. Since variation in the genome is a dynamic process, it is necessary that a multiplex assay uses the most conserved region representing all the strains/variants for detection of the pathogen in body fluid. In the case of hepatitis viral infections, studies have reported a distinct conserved region that has been used as a target for amplification of each individual viral genome[4,20,21]. Table 1 shows the list of target regions used in various studies on multiplex qPCR assays developed for hepatitis viruses. The 5′ untranslated region (UTR) was reported to be the main target template in HAV, HCV, and HGV[20,22]. It was based on the availability of most conserved sequence in the 5′ UTR for amplification purpose. Similarly, S-gene or X-gene was used for HBV, ribozyme-1 gene for HDV, and open reading frame (ORF)-2 or ORF-3 region for HEV. Different studies have reported different sequences as templates in these selected conserved regions, though, there was very little information provided about the exact location of the sequences used.

Designing of primers and probes

After deciding which conserved region and location of the sequence were to be used as template, it is important to design the primers and probes for their use in the development of qPCR[23]. The selection of the primer is based on its specificity with the target template. At the same time, its length, melting temperature, GC content, 3′ end stability, sequence complexity, and location in the target sequence determine the length and melting temperature of the amplicon produced and the amplification efficiency of the assay[23,24]. Notably, the choice of chemistry and probe design are at the liberty of the user's interest, with numerous options available to them[24]. During selection of chemistry and probe, one needs to determine whether to quantify DNA, profile mRNA, or perform allelic discrimination assays[25].

Real-time PCR and melting curve analysis (MCA) are good techniques for quantifying nucleic acids, detecting mutations, and conducting genotyping analysis. These methods often use TaqMan probes[26], Molecular beacons[27], Sunrise primers[28], Scorpion primers[29], and Light-up probes[30]. An alternative to probe-based methods is the use of DNA intercalating dyes that bind to double-stranded DNA. These dyes include ethidium bromide[31] and SYBR Green I[32,33]. However, certain drawbacks limit the use of SYBR Green I for resolving multiplex PCR based on MCA[34]. Other alternative dyes, such as BEBO[35], YO-PRO-1[36], LC Green[37], and SYTO-9[38,39] have also been tried for use in real time PCR. Table 2 provides a brief review of various chemistries/dyes offering several options for their use in qPCR assay developed for different purposes. Studies for detecting and genotyping hepatitis viruses with qPCR have reported different sets of dyes based on choice and their availability[40]. However, most of the studies conducted have reported a frequent use of hydrolysis probes despite many options available. This information is available in the data[41-71] compiled in Table 3.

The probe-based assays (*e.g.,* TaqMan assays)[72] began to gain attention in mid-1990s with the development of quenched, fluorescent probes[73,74] and the commercialization of real-time thermal cyclers[26,75]. TaqMan (also known as Fluorogenic 5′ nuclease assay) probes contain two dyes, a reporter dye (*e.g.,* 6-FAM) at the 5′ end and an acceptor dye at 3′ end, usually tetramethyl rhodamine (TAMRA). Recently, TAMRA fluorescent acceptor quencher dye was substituted with a non-fluorescent quencher, *e.g*., Black Hole Quencher[76]. The proximity of the quencher to the reporter in an intact probe quenches the fluorescence signal of the reporter dye through fluorescence resonance energy transfer. During amplification, the 5′ to 3′ nucleolytic activity of Taq polymerase cleaves the probe between the reporter and the quencher only if the probe hybridizes to the target. The probe fragments get displaced from the target, separating the reporter dye from the quencher dye, resulting in increased emission of fluorescence. Floating TaqMan probes are quenched due to random coiling in solution, where fluorophore- and quencher-labeled ends come together[77]. In contrast, Molecular Beacon probes are oligonucleotides designed in a way to induce hairpin formation and produce the quenched state[78]. TaqMan and Molecular Beacon probes have been shown to be less effective in discriminating closely related targets, as in single nucleotide polymorphisms, drug-resistant mutants, and somatic cancer mutations[79,80]. However, molecular beacons are useful in situations where it is not possible to isolate probe-target hybrids from

Table 2 Chemistries/Dyes used in qPCR assays

S. NO.	Class	Types	Structure	Mechanism of action	Advantages	Applications
1	DNA binding dyes	Ethidium Bromide, SYBR Green, SYBR Gold, YO-PRO-1, SYTO, BEBO, BOXTO, EvaGreen	Intercalating dyes	Bind to the minor groove of dsDNA during amplification	Inexpensive Easily available	Pathogen detection Gene expression SNP detection Genotyping
2	Fluorophore labeled oligonucleotide	*Primer probes*				
		Hairpins: Scorpions, Ampliflour, LUX	Loop based oligonucleotides	Bind to target during denaturation with emission of fluorescence	Inexpensive, Prevent formation of primer dimer, Less background signals	Pathogen detection Genotyping SNP allelic discrimination Mutation detection
		Cyclicons	Cyclic structure with reporter at 3′ end and quencher at 5′ end	Reporter and quencher in close proximity with energy transfer *via* FRET quenching. Their separation results in fluorescence emission during amplification	Inexpensive Less contamination Less background signals	Pathogen detection Genotyping SNP allelic discrimination Mutation detection
		Angler	Probe with DNA sequence bound to reverse primer through a HEG linker	During annealing step, DNA polymerase does extension of 3′ end reverse primer. Later on, SYBR Gold dye intercalates in dsDNA emitting fluorescence	Highly specific	Gene expression Pathogen detection SNP detection Genotyping
		Probes				
		Hydrolysis Probes: TaqMan probes, MGB-TaqMan, Snake assay	Oligonucleotide with reporter at 5′ and quencher at 3′ end	Probe is degraded by 5′ to 3′ exonuclease activity of DNA polymerase generating fluorescence during extension	Design and synthesis easy	Microarray validation Pathogen detection SNP allelic discrimination Mutation detection
		Hybridization probes: Hybprobes, Molecular Beacon, HyBeacon, MGB Probes	A pair of oligonucleotides having reporter dye on first and quencher on second oligonucleotide	Binding to target during hybridization and annealing brings fluorophore into proximity producing fluorescence by FRET	Design and synthesis quick and easy	Microarray validation Pathogen detection Viral/Bacterial genotyping SNP allelic discrimination Mutation detection
		Nucleic acid analogues				
		PNAs, LNAs, ZNAs Non-natural bases	Intercalating/inserting dyes	Identical to conventional oligonucleotides	Resistant to nuclease and proteases activity	Discriminate between DNA and cDNA in prokaryotes

All above details were collected from report published in *Clinica Chimica Acta* 2015; **439**: 231-250[25]. SNP: Single nucleotide polymorphisms.

an excess of the hybridization probes, for example in sealed tubes or within living cells[81]. An effective probe requires a careful balancing act based on melting temperature (Tm) and, therefore, repeated design and testing are needed to develop an effective probe[82]. Available evidence suggests that the use of TaqMan probes in qPCR assays for hepatitis viruses provide a good balancing act.

Designing tools

Today, several designing tools are available to guide the design of qPCR assays and analyze resulting quantitative data. Many of them are available online, and some are provided with qPCR instruments from different manufacturers[83]. Some important tools include Primer3, Primer-BLAST, PerlPrimer, FastPCR software, IDTSciTools, and UniPrime[84-89]. In addition, some of them have programming to analyze the secondary structure of primers. MP primer is used to design primers for multiplex PCR assays[90]. The Minimum Information for Publication of qPCR Experiments (MIQE) guidelines also provide clear instructions on the steps that are important for qPCR assay design[91]. Several research companies offer help for designing primers and probes with use of their designing tools. The studies reported in this article demonstrate a liberal use of tools without any specific need or choice affecting the results.

Instruments used in multiplex qPCR assay

Various types of advanced technology-based equipment for multiplex qPCR assays with analysis of amplified products are available globally. A list of the instruments used with their brands in various studies conducted on qPCR for viral hepatitis is shown in Table 3. With increasing advances in technology, the number of filters and, accordingly, the resolution of the amplification curve during the PCR assay have also increased. Now it is possible to detect/discriminate more pathogens or allelic/mutational changes[92,93] in a

Table 3 Global status of multiplex qPCR developed for hepatitis viral infections with and without other pathogens

No.	Assay systems	Instruments used	Group of pathogens detected		Types of chemistries/ detection methods used	Ref.
			Hepatitis viruses	Other pathogens		
1	Multiplex real time PCR	Mx4000 (Stratagene)	HBV, HCV	HIV type-1, T. pallidum	TaqMan-LNA probe	[21]
2	Multiplex real time PCR	Light cycler 480 (Roche)	HEV genotypes	-	N.A.	[41]
3	Real time PCR assay	ABI 7500 (Applied Biosystems)	HAV, HBV, HCV, HDV, HEV	-	TaqMan Array card	[42]
4	Multiplex qPCR assay	Light cycler 480 (Roche)	HBV, HDV	-	TaqMan probe	[43]
5	Multiplex qPCR assay	ABI 7500 (Applied Biosystems)	HAV, HEV	-	Hydrolysis probe	[22]
6	Multiplex qRT-PCR	N.A.	HAV	Norovirus genotypes 1 and 2	TaqMan probe	[44]
7	Multiplex ligation dependent probe real time PCR	Rotor-GeneQ (Qiagen)	HBV mutants	-	TaqMan probe MLPA probe	[45]
8	Multiplex real time RT-PCR	N.A.	HEV genotypes	-	N.A.	[46]
9	Multiplex qPCR	N.A.	HBV genotypes	-	SYBR Green	[47]
10	Multiplex Real time PCR	N.A.	HAV	Norovirus, Rotavirus, Coxsackievirus	TaqMan probe	[48]
11	Multiplex Real time PCR	Light cycler 2.0 (Roche)	HAV, HBV, HCV and HEV	-	FRET probe	[15]
12	Multiplex RT-PCR	ABI 2720 (Applied Biosystems)	HCV	HIV type-1	SYBR Green I	[8]
13	Multiplex qPCR	N.A.	HAV, HEV	Entero and Adeno-viruses	N.A.	[49]
14	Multiplex Real-Time PCR Assay	CFX96 (Bio-Rad)	HAV, HBV, HCV	-	READ technology based fluorophore	[4]
15	RT PCR assay	Smart cycler Ⅱ (Cepheid)	HBV, HCV	-	TaqMan probe	[50]
16	Duplex real time PCR	ABI 7500 (Applied Biosystems)	HBV variants	-	Hydrolysis probe	[51]
17	Multiplex RT PCR	N.A.	HCV subtyping	-	Electrophoresis	[52]
18	Multiplex qPCR	N.A.	HBV genotypes	-	N.A.	[53]
19	Multiplex qPCR	N.A.	HCV	HIV type-1	SYBR Green I	[54]
20	Duplex real-time RT-PCR	ABI Prism system (Applied Biosystems)	HCV variants	-	Hydrolysis probe	[55]
21	Multiplex real time PCR	N.A.	HAV	Norovirus genotypes 1 and 2	N.A.	[56]
22	Duplex real-time qRT-PCR	ABI Prism 7000 (Applied Biosystems)	HAV	MS2 bacteriophage	MGB-TaqMan probe	[57]
23	Multiplex TaqMan RT-qPCR system	MX30005P (Stratagene)	HEV	FCV	TaqMan probe	[58]
24	Multiplex real time PCR	ABI 7300 (Applied Biosystems)	HBV genotypes	-	TaqMan probe	[59]
25	Real time PCR	N.A.	HBV genotypes	-	TaqMan probe	[60]
26	Multiplex real time PCR	Mx3005P (Stratagene)	HEV	FCV	TaqMan probe	[61]
27	Multiplex RT PCR assay	ABI Prism 7500 (Applied Biosystems)	HCV	PDV	MGB hybridization probe	[62]
28	Multiplex qPCR assay	N.A.	HBV	B19, HHV-8, EBV, CMV, VZV	N.A.	[63]
29	Multiplex qPCR	N.A.	HBV, HCV	HIV type-1	SYBR Green I	[16]
30	Multiplex Real Time PCR	ABI 7500 (Applied Biosystems)	HBV mutants	-	LNA probes with SYBR Green I	[64]
31	Microarray multiplex assay	ABI Prism 7700 (Applied Biosystems)	HBV, HCV	HIV type-1	Oligonucleotide array labeled with Cy5 and Cy3	[65]
32	Real time multiplex PCR	N.A.	HAV	Entero and Adeno-viruses	Probes labeled with FAM, R6G, ROX, Cy5	[66]
33	Multiplex real time RT-PCR	LightCycler (Roche)	HCV	HIV type-1	SYBR Green	[67]
34	Real time multiplex PCR	icycler iQ (Bio-Rad)	HCV variants	-	TaqMan probes	[68]
35	Multiplex real-time RT PCR	ABI 7000 (Applied Biosystems)	HCV genotypes	-	Primer probes	[69]
36	Multiplex real-time qPCR	Mx4000 (Stratagene)	HBV, HCV	HIV type-1	TaqMan probes	[70]
37	Automated multiplex PCR	ABI Prism 7700 (Applied Biosystems)	HBV, HCV	HIV type-1	TaqMan probes	[71]

single step multiplex assay. The choice of instrument is more a function of availability, without much difference in their analytical qualities. Multiplex qPCR assays developed for hepatitis viruses may use any brand, depending on a match between the number of component pathogens to be detected and the filters

available for detection. Other features of equipment do not seem to affect the results.

Optimization of protocol

For each pathogen used as a component in the multiplex assay, a carefully developed singleplex assay is needed. The design of primers and probes is dictated purely by the nature of the target template and clear guidelines for amplification. This exercise is followed in order to prepare a record of common amplification conditions noted in singleplex assays and for their application as such in multiplex assays. The multiplex protocol is reframed in a way to have minimum possible deviations from the working protocol of the singleplex assay. During the multiplex assay, the possibility of cross interaction/interference among different molecules is quite likely and may cause unsuccessful amplification. This interaction may or may not occur, but it has to be worked out cautiously in each multiplex assay.

There have been reports on singleplex as well as multiplex assays developed for detection of some hepatitis viruses and their genotypes[16] (Table 3). Such a study was conducted at our research center where a multiplex assay was developed for simultaneous detection of hepatitis virus A, B, C, and E[15]. These viruses are frequently prevalent in India, posing a serious problem, causing incidences of both sporadic and epidemic hepatitis from time to time[94,95]. The use of singleplex followed by the development of multiplex assay in these cases does not show many changes in the experimental protocol. This implies that the amplification protocol of individual viruses is not influenced during multiplex assays. We noted a clear amplification curve on the screen during multiplex assay that was the same exact pattern noted during singleplex assay[15].

Table 3 shows the global status of multiplex assays used for analyzing hepatitis viruses with or without other pathogens[41-71]. In all these assays, viral amplification by the simultaneous presence of other pathogenic genomes was indicated. An overall survey of the experimental designs reported in multiplex assays indicated that standard conditions of reverse transcription, denaturation, annealing, and extension temperature were followed without much deviation from the singleplex protocol.

MIQE guidelines

The guidelines published by Bustin *et al*[91] in 2009 clearly defined the terms used and steps necessary to design the experiments for developing qPCR assay. Since 2009, many published reports in the area of viral hepatitis on multiplex qPCR were found to follow these guidelines and give interpretation of results referring to terminology and definitions outlined there. The guidelines state that multiplexing expands power of qPCR analysis but needs documentation for accurate quantification of multiple targets in a single tube assay.

ASSESSMENT OF SENSITIVITY AND SPECIFICITY

Generation of standard curve

In order to generate a standard curve for each hepatitis virus, the standard control that includes the conserved region targeted for amplification/ detection is synthesized artificially and cloned into a suitable vector (*e.g.,* pUC 57)[15] using cloning kits. These standards are used as a template for standardization of amplifications. The copy number of standard plasmids is calculated using their concentration and the size of linearized plasmids. Each standard template is added to PCR mix (Tris-HCl, KCl, $MgCl_2$, four dNTPs, primers, and Taq DNA polymerase in a suitable concentration ratio), and PCR is performed under standardized conditions. For generation of the standard curve, a 10-fold serial dilution of each standard plasmid (10^1-10^8 copies/μL) is prepared and run in triplicate. At the end, data are analyzed by an automatic system that generates a standard curve[21]. The standard curves are used to quantify the amplification product and to assess the linear dynamic range using 10-fold dilution series of standard plasmid of each individual virus. One specimen standard plot is shown in Figure 1, which was prepared during development of quadruplex qPCR for hepatitis virus A, B, C, and E. Such plots are used to calculate copy number of individual template using correlation coefficient and Y-intercept value based on regression analysis.

Standard curve showing amplification plots of 10-fold serial dilution of HAV template using standard cloned plasmids. Such standard curves are generated from the amplification plots run in triplicate and show a linear dynamic range. The correlation coefficient and the slope of each standard plot are shown in the figure.

Assessment of sensitivity

Using the standard curve prepared above, now it is possible to assess the sensitivity and determine the linear dynamic range of an individual virus. Moreover, observed Ct values may be used to calculate the exact copy number of virus in an unknown specimen[96,97]. Based on the data collected from various studies, including our study[15], it has been noticed that the linear dynamic range of each individual hepatitis virus usually falls in the range 10^1-10^8 copies/μL.

Assessment of specificity

The specificity of qPCR assay is assessed by evaluating sera from healthy controls and patients with unrelated diseases negative for hepatitis markers by serology and all other NAT based techniques. Negative results from these sera and clear positive signals from serologically positive hepatitis sera demonstrate the high level of specificity of qPCR. To date, all studies on qPCR demonstrate the assay to be specific[15,22]. In

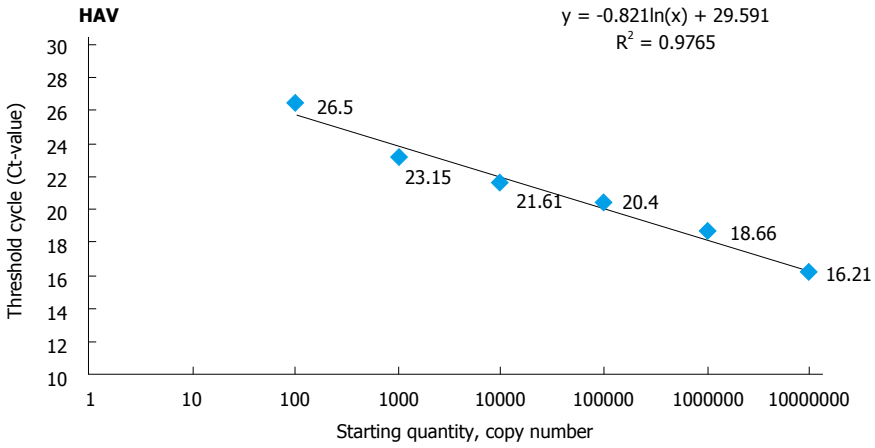

Figure 1 Standard curve showing amplification of hepatitis A virus[15]. HAV: Hepatitis A virus.

reports on viral hepatitis, qPCR assays demonstrated high specificity with a very low chance of false positive results[19,71].

MULTIPLEX qPCR IN RELATION TO OTHER ASSAYS

The multiplex qPCR assays were developed and used both for comparison as well as in combination with other molecular technologies to improve the sensitivity for detection of the viral genome[16,98]. Various other assay systems were also developed for simultaneous detection of HBV, HCV, and human immunodeficiency virus in addition to multiplex qPCR. The status of multiplex qPCR assay was assessed in comparison to other molecular techniques used for detection and genotyping of viruses, including hepatitis viruses. The other assay systems included flowcytometric microsphere based hybridization assay[99], transcription-mediated amplification (TMA)[100], and nucleic acid sequence based amplification (NASBA)[101]. Comparatively, TMA was reported to be an equally sensitive technique. However, when comparing qPCR with NASBA and TMA for the detection of hepatitis viruses, the level of sensitivity of TMA was found to be associated closely with qPCR[100]. Of course, qPCR assay was reported to be faster, more economic, and easier to perform compared to all other assays evaluated.

FUTURE AND LIMITATIONS OF MULTIPLEX qPCR

Multiplex qPCR assays are proving to be very good analytical and diagnostic procedures in medicine. Recently, these assays have been successfully used for both basic research and clinical applications[42,102]. Although the practice of doing separate assays for separate pathogens, including hepatitis viral markers, are still in place, the use of the multiplex assay is seen to be beneficial in terms of time and overall cost involved. Moreover, multiplex assays, when used for quantification of HCV- RNA, were found to resolve many problems with real time monitoring of the amplification process. In fact, in multiplex qPCR assays, real time PCR makes quantification of DNA and RNA of different organism more precisely and with better reproducibility because it depends on the threshold cycle value determined during the exponential phase of PCR rather than on end points[103]. In addition, these assays report a direct relationship between starting template copy number and the number of cycles required to get a positive signal. In this manner, real time qPCR appears to be a good option for laboratory diagnosis of viral hepatitis, both for screening as well as for the final diagnosis of suspected cases of viral hepatitis infections.

CONCLUSION

Based on the information compiled in the present review, there is an increasing trend/interest in the diagnostic area towards the development and use of multiplex qPCR assay for the simultaneous detection of hepatitis viruses or their subtypes in sera samples. Several studies have been conducted in last few years that clearly demonstrate the preferable use of qPCR over other techniques in the area of viral hepatitis. This technique has been used to detect hepatitis viruses in combination with various other viral and non-viral pathogens and reported to be a sensitive, fast, and cost-effective technique compared to other multi-step assay procedures. The use of multiplex qPCR in genotyping of hepatitis viral subtypes also provides great help in serotype detection. To date, multiplex qPCR has been successfully employed for the simultaneous detection of hepatitis virus A, B, C, D, and E and genotyping of their strains. It appears to be a good tool for screening blood donor samples in blood banks for hepatitis viruses. Moreover, a single step multiplex qPCR assay allows for an early diagnosis

and timely treatment of patients with viral hepatitis. Several studies in this field are in progress, with more important information likely to be available until the next such update is necessary.

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REVIEW

Advanced imaging techniques in the therapeutic response of transarterial chemoembolization for hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is one of the major causes of morbidity and mortality in patients with chronic liver disease. Transarterial chemoembolization (TACE) can significantly improve the survival rate of patients with HCC and is the first treatment choice for patients who are not suitable for surgical resections. The evaluation of the response to TACE treatment affects not only the assessment of the therapy efficacy but also the development of the next step in the treatment plan. The use of imaging to examine changes in tumor volume to assess the response of solid tumors to treatment has been controversial. In recent years, the emergence of new imaging technology has made it possible to observe the response of tumors to treatment prior to any morphological changes. In this article, the advances in studies reporting the use of computed tomography perfusion imaging, diffusion-weighted magnetic resonance imaging (MRI), intravoxel incoherent motion, diffusion kurtosis imaging, magnetic resonance spectroscopy, magnetic resonance perfusion-weighted imaging, blood oxygen level-dependent MRI, positron emission tomography (PET)/computed tomography and PET/MRI to assess the TACE treatment response are reviewed.

Key words: Blood oxygen level-dependent; Computed tomography perfusion imaging; Chemoembolization; Diffusion kurtosis imaging; Diffusion-weighted imaging; Hepatocellular carcinoma; Magnetic resonance perfusion-weighted imaging; Intravoxel incoherent motion; Magnetic resonance spectroscopy

Core tip: Imaging studies play an important role in the evaluation of the response to transarterial chemoembolization treatment. The use of imaging to examine changes in tumor size to assess the response of solid tumors to treatment has been controversial. In recent years, the emergence of new imaging technologies has made it possible to observe the response of tumors to treatment prior to any morphological changes. In this article, we present a summary of the most recent information on the role of imaging in assessing the treatment response in hepatocellular carcinomas.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the major causes of morbidity and mortality in patients with chronic liver disease. Due to the undetected onset of liver cancer, the majority of patients receiving treatment are already in the advanced stage and are no longer candidates for surgical resection. Transarterial chemoembolization (TACE) involves the local infusion of a mixture of chemotherapeutic agents, blocks the blood supply to cancerous lesions and induces ischemia and necrosis in the tumor tissue, thereby significantly improving the survival rate of patients with liver cancer[1-4]. Currently, TACE has been recommended as the standard treatment for patients with stage B (Barcelona Clinic Liver Cancer staging) HCC[5,6]. The assessment of the response of HCC to TACE treatment affects not only the evaluation of the therapeutic efficacy but also the development of the next step in the treatment plan, including the time and frequency of repeated chemoembolization[7]. The previous World Health Organization and Response Evaluation Criteria in Solid Tumors criteria for evaluating the response of solid tumors to treatment depended on the measurement of tumor size[8]. The use of conventional imaging techniques to examine changes in tumor size to assess the response of solid tumors to treatment has been controversial, as many HCC treatments act by inducing tumor necrosis or by reducing vascularity, which is not necessarily accompanied by tumor shrinkage even when response occurs; notably, some tumors clearly respond to treatment but show no remarkable changes in size[9,10]. In recent years, the assessment of tumor viability has attracted increasing attention. The modified Response Evaluation Criteria in Solid Tumors criteria recommended by the European Association for the Study of the Liver consider the treatment factors leading to tumor necrosis and define the lesions that uptake a contrasting agent in the arterial phase as the surviving tumor after treatment[11]. In recent years, the emergence of new imaging technologies has made it possible to observe the response of tumors to treatment prior to any morphological changes. In this article, studies reporting advances in the use of computed tomography perfusion imaging (CTPI), diffusion-weighted magnetic resonance imaging (DWI), intravoxel incoherent motion (IVIM), diffusion kurtosis imaging (DKI), magnetic resonance spectroscopy (MRS), magnetic resonance perfusion-weighted imaging (MR PWI), blood oxygen level-dependent magnetic resonance imaging (BOLD MRI), positron emission tomography/computed tomography (PET/CT) and PET/MRI to assess the response to TACE treatment are reviewed.

COMPUTED TOMOGRAPHY PERFUSION IMAGING

Lipiodol is an ideal embolic agent commonly used in TACE treatment for HCC, and studies have shown that the deposition of lipiodol in the lesions is correlated with antitumor effects[12]. Conventional CT scanning has been widely used in the evaluation and follow-up of the efficacy of TACE treatment for HCC. Though CT can be used to visualize the distribution of lipiodol within the lesions, the high density deposition of lipiodol in tumor tissue can significantly affect the judgment of the viability of the tumor by CT.

CTPI not only clearly shows anatomy of the liver but also reflects changes in liver hemodynamics by allowing the quantitative analysis of blood perfusion in the liver tissue. CTPI performs continuous dynamic scans on selected slices while a contrast agent is intravenously injected, resulting in a curve that reflects the density changes of each pixel within the slice over time (time-density curve). A variety of mathematical models are then used to calculate the various perfusion parameters of the tissues and organs to evaluate the blood perfusion status[13-16] (Figure 1). The main parameters measured by CTPI include hepatic arterial perfusion (HAP), hepatic portal perfusion (HPP), total liver perfusion (TLP), hepatic arterial perfusion index (HAPI), hepatic portal perfusion index (HPPI), blood volume (BV) and mean transit time (MTT). Early CTPI scans used a single-slice continuous dynamic scan mode, but with progress in the development of multi-slice CT and software technology, CTPI has advanced from single-slice perfusion scans to multi-slice and same slice dynamic CT perfusion scans. Currently, spiral CT involving 64 or more slices can be used to conduct full-size liver perfusion scans with greatly improved temporal and spatial resolutions, which allows for the acquisition of more comprehensive hemodynamic information in a single scan. Moreover,

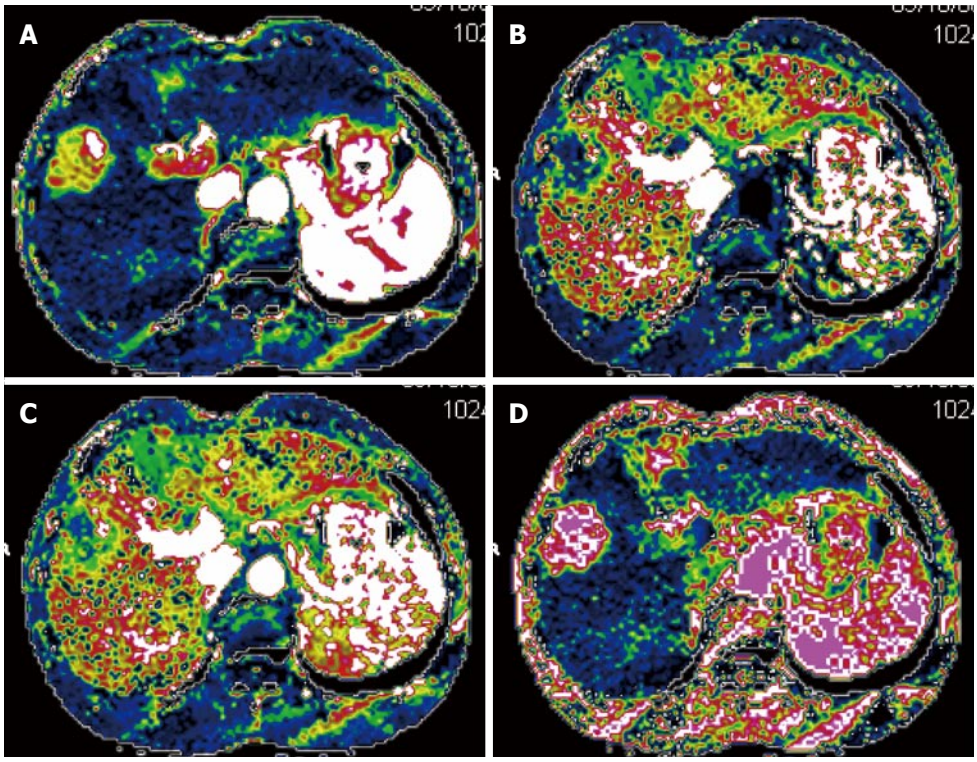

Figure 1 Seventy-year-old male patient with hepatocellular carcinoma. Axial perfusion images of the tumor before transarterial chemoembolization were created by maximum slope method. The tumor showed an increased hepatic arterial perfusion and decreased hepatic portal perfusion compared with the normal parenchyma. The values of hepatic arterial perfusion, hepatic portal perfusion, total liver perfusion and hepatic arterial perfusion index were 0.512 mL/min.mL, 0.226 mL/min.mL, 0.738 mL/min.mL and 69.4%, respectively. A: Image of hepatic arterial perfusion; B: Image of hepatic portal perfusion; C: Image of total liver perfusion; D: Image of hepatic arterial perfusion index.

lesions distant from the hilum can also be measured using CTPI, which has further promoted the clinical application of this technique[17-19].

The blood supply to HCC is one of the main factors affecting the efficacy of TACE treatment[20]. Increased blood supply to the HCC is associated with greater lipiodol accumulation after TACE treatment, whereas reduced blood supply to the HCC results in rather small amounts of lipiodol deposition in the treated lesions[21].

Many investigators have examined the effectiveness of CTPI in evaluating the response of HCC to TACE treatment, suggesting that CT perfusion imaging can accurately measure blood perfusion to the tumor and thus could be used to evaluate the response to TACE therapy[22-27]. Chen *et al*[22] assessed the changes in the CT perfusion parameters pre- and post-TACE in thirty-nine HCC patients in different treatment response groups. In the partial response (PR) treatment response group, the HAP, hepatic arterial fracture (HAF) and hepatic blood volume (HBV) of viable tumors post-TACE were reduced compared with their pre-TACE values. In the stable disease (SD) group, however, none of the CT perfusion parameters were significantly different pre- and post-TACE. In the progressive disease (PD) group, the post-TACE values for HAP, HAF, portal vein perfusion (PVP) and hepatic blood flow (HBF) of viable tumors were significantly increased compared to the pre-TACE values. These results indicated that changes in the CT perfusion parameters of viable tumors are correlated with responses of HCC to TACE, which can be feasibly monitored using CTPI. Reiner *et al*[23] studied sixteen patients with HCC who received CT liver perfusion during the treatment planning stage prior to transarterial radioembolization with Yttrium-90 (90Y) microspheres. The results showed that when responders were compared to non-responders, the 50th and 75th percentiles of arterial perfusion were significantly different and that the response to therapy could be predicted with a sensitivity of 88% and specificity of 75%. Our own studies[9,21] have shown that the CT perfusion parameters of HCC (HAP, TLP and HAPI) significantly decreased after TACE treatment[9] and that the blood perfusion parameters of the HCC lesions were correlated with post-TACE lipiodol deposition. Moreover, increased amounts of blood perfusion were associated with the increased deposition of lipiodol, and vice versa[21]. On the CT perfusion images, the areas with densely deposited lipiodol in the residual lesions in cases with complete or partial response (PR) displayed the complete absence of blood perfusion[9].

These results show that CTPI can be used to accurately measure the changes in perfusion parameters after TACE treatment for HCC and to evaluate the response to TACE therapy prior to changes in tumor size. CTPI can also be used to predict the efficacy of TACE therapy for HCC, to help select appropriate patients for TACE therapy and to develop individualized

treatment programs.

C-arm CT has emerged in recent years and can quantitatively measure the blood volume (BV) changes in tumor tissues. This technique has dramatically increased the convenience of assessing the response to TACE therapy[28-30]. Peynircioğlu *et al*[30] performed radioembolization (n = 21) or TACE (n = 13) treatment on thirty-four patients with HCC and used C-arm CT to measure the tumor BV before and after treatment. These cases were compared to ten cases in which perfusion imaging was performed using multidetector computed tomography (MDCT). The results showed that the mean BV of fourteen tumor lesions in the ten MDCT perfusion patients was highly correlated with the BV values obtained with C-arm CT. After treatment with TACE or radioembolization, the BV values decreased significantly, suggesting that the quantitative BV measurements obtained using C-arm CT are well-correlated with those obtained using MDCT; thus, C-arm CT is a promising tool for monitoring perfusion changes during hepatic arterial embolization. Currently, C-arm CT is mainly used to measure BV, but with the further development of this method, additional parameters can be used in the evaluation of TACE in the clinical treatment of HCC.

The main shortcoming of CTPI is that perfusion CT studies increase radiation exposure. In the future, with improvements to the equipment and technology, the radiation dose will be reduced.

DIFFUSION-WEIGHTED MRI

DWI is currently the only non-invasive imaging technique that can detect the free diffusion motion (Brownian motion) of water molecules in living tissue. Detecting the free diffusion motion of water molecules in the human body enables magnetic resonance at the molecular level. DWI not only reflects the dispersion characteristics of various tissues but also enables quantitative analyses of the microscopic structures and functional changes of tissues and organs. Though DWI has mainly been used in studies of central nervous system diseases[31-33], this technique is increasingly being applied to abdominal examinations[34-42]. At present, the commonly used single-time spin echo-echo planar imaging (SE-EPI) can image in rapid sequence and only takes 20-30 s to complete a liver scan[43], prompting the application of SE-EPI in the diagnosis and treatment of liver diseases.

DWI enables quantitative analyses by measuring the apparent diffusion coefficient (ADC) value. Thus, this technique can be used in assessing the effectiveness of TACE treatment for HCC[44-47]. After TACE therapy for HCC, the tumor cells undergo necrosis and decrease in number, the gaps between cells enlarge, and structures such as the cell membranes are damaged or dissolved, leading to enhanced water diffusion capacity and an increased ADC value. When the tumor survives or recurs, however, the ADC value does not increase or decrease. Bonekamp[44] used TACE therapy to treat seventy-one HCC lesions in forty-eight patients and performed MRI scans before the TACE treatment and one and six months after the treatment to monitor the ADC and venous enhancement (VE) as the tumor changed in size. The results demonstrated that thirty HCC lesions showed PR, thirty-five showed SD, and six showed PD 6 mo after TACE. Increase in ADC and decrease in VE 1 mo after TACE were significantly different between PR, SD, and PD. Yu *et al*[45] used enhanced MRI and DWI scans in twenty-three patients with liver cancer who had received TACE treatment, finding a total of twenty-three recurrent nodules in sixteen cases; the overall sensitivity in DWI was increased from 85.0% to 92.0%, though the specificity was decreased from 65.0% to 50.0%. The pre-TACE tumor ADC can be used to predict the response of HCC to TACE treatment. Mannelli *et al*[47] conducted DWI scans on thirty-six patients receiving TACE treatment for HCC and found that HCCs with poor and incomplete responses to TACE had significantly lower pre-treatment values of ADC and lower post-TACE values of ADC compared to HCCs with good or complete responses.

The shortcomings of DWI include EPI-related artifacts, such as deformation artifacts. Moreover, the ADC values of benign and malignant nodules in the liver overlap to some extent, and discriminating between these values requires a combination of medical history and other test results. Many factors that influence the ADC value, including the MR device, scan parameters (TR and TE), the b-value and the ROI, should be investigated in the future.

INTRAVOXEL INCOHERENT MOTION MR IMAGING

During DWI imaging, the b-value (a gradient factor) determines the sensitivity of the diffusion motion of water molecules in the tissue under analysis while affecting the accuracy of the ADC value. A low b-value enables the acquisition of images with a high SNR but lowers the sensitivity to the diffusion motion, resulting in a higher impact exerted by the blood perfusion on the DWI imaging. Under a high b-value, blood perfusion only has a small impact on DWI imaging, but the tissue contrast is decreased, leading to poor image quality[35,48-50].

IVIM, a multi-b-value diffusion-weighted imaging approach based on the principle of DWI, uses quantitative indicators to show the molecular diffusion and microperfusion of the local capillary network in lesions. The commonly used parameters include the true molecular-diffusion coefficient (D), the perfusion-related diffusion coefficient (D*) and the perfusion fraction (f). Compared with DWI, IVIM better reveals the diffusion effect of water molecules within a lesion

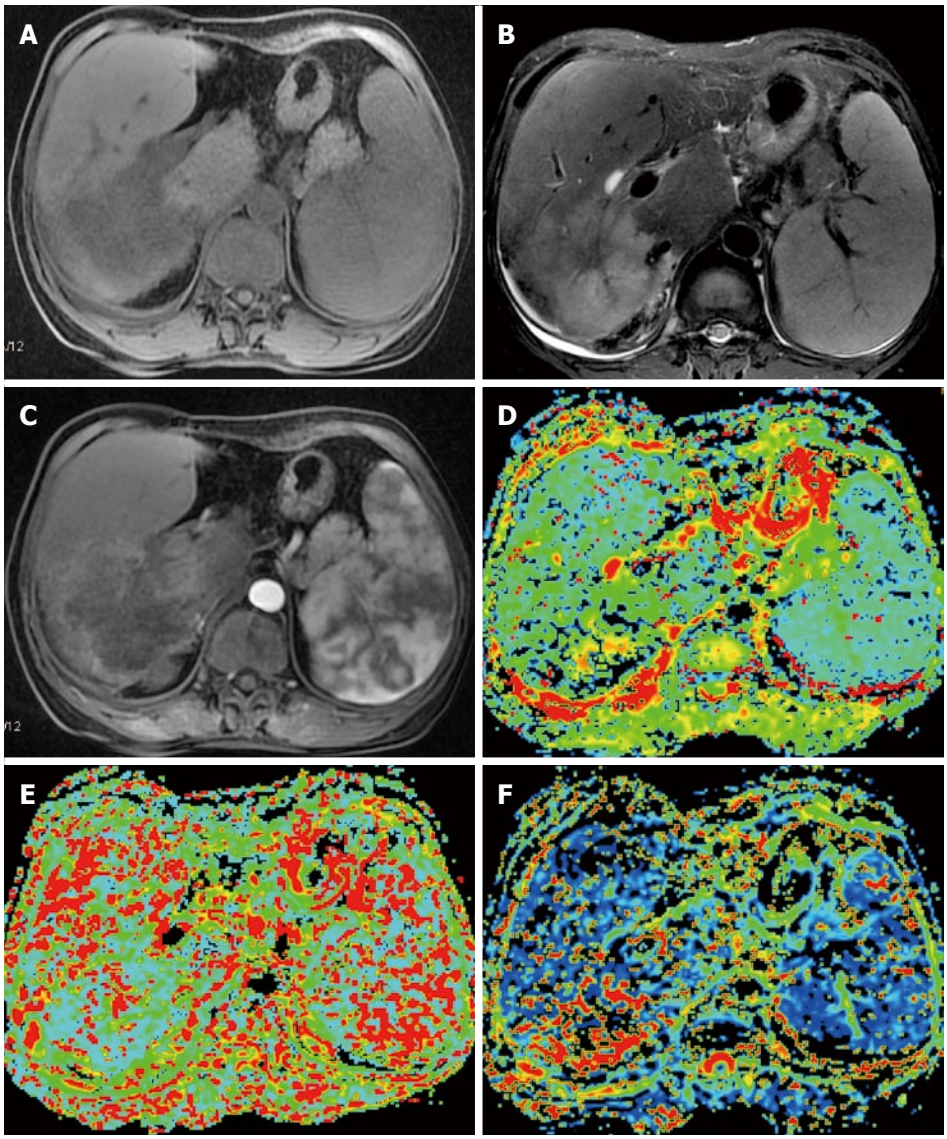

Figure 2 Fifty-three-year-old female patient with hepatocellular carcinoma in the right lobe of the liver. A: Axial T1-weighted image shows a hypointense mass lesion; B: Axial T2-weighted image shows a hyperintense mass lesion; C: Contrast-enhanced MRI during the arterial phase showing lesion enhancement; D: Mapping of the estimated value of the *D* parameter. The average value in the lesion ROI was $D = 1.22 \times 10^{-3}$ mm^2/s; E: Mapping of the estimated value of the *D** parameter. The average value in the lesion ROI was $D^* = 20.6 \times 10^{-3}$ mm^2/s; F: Mapping of the perfusion fraction (f) with a value of 19.6%.

and is thus more conducive to making judgments regarding the nature of liver lesions[51-55]. Watanabe *et al*[51] performed IVIM imaging on a total of 120 liver lesions (including 34 metastases, 32 HCC, 33 hemangiomas and 21 liver cysts) in seventy-four patients and showed that the mean D and ADC values of the benign lesions were greater than those of malignant lesions. The area under the ROC curve for the ADC values was significantly greater than that for the D values, which enabled the differentiation between benign and malignant lesions. When an ADC cut-off value of 1.40 was applied, the sensitivity and specificity for the detection of malignant lesions were 89% and 98%, respectively. Other studies have shown similar results[52-55] (Figure 2).

The applications of IVIM in cancer treatment evaluation have mainly focused on radiotherapy or chemotherapy in head and neck cancers and in breast cancer[56-67]. In recent years, some investigators have applied IVIM in the anti-angiogenesis therapy of liver cancer (including metastatic liver cancer) or radiofrequency ablation therapy[68-73]. Guo *et al*[71] investigated the use of IVIM-DWI to monitor the responses of VX2 tumors to radiofrequency ablation (RF Ablation) therapy in 10 VX2 tumor-bearing rabbits, showing that the IVIM-DWI derived f, D and D* parameters have the potential to indicate the response to therapy immediately after the RF ablation treatment. Shirota *et al*[72] evaluated the association between the therapeutic outcomes of sorafenib for advanced HCC and the parameters of IVIM. Though the true diffusion coefficient (DC) of responders at baseline was significantly higher than that of the non-responders, no significant differences were found in the

other parameters between these two groups. These results indicated that the DC before treatment may be a useful parameter for predicting the therapeutic outcome of using sorafenib to treat advanced HCC.

The use of IVIM to assess the response to TACE treatment in liver cancer has rarely been reported. Park *et al*[74] performed IVIM-DWI and Gd-EOB-DTPA-enhanced MRI scans before TACE therapy in forty-four cases of HCC and conducted CT scans after the TACE treatment. The patients were divided into two groups, the lipiodol good uptake (LGU) group and the lipiodol poor uptake (LPU) group, based on lipiodol deposition, and the results showed that both the arterial enhancement ratio derived from the contrast enhanced MRI and the D* values derived from IVIM-DWI were significantly higher in the LGU group than in the LPU group, indicating that the parameters of IVIM could help to predict lipiodol uptake.

DIFFUSION KURTOSIS IMAGING

The theoretical basis of the DWI and IVIM technologies is that the diffusion of water molecules *in vivo* assumes a normal distribution. In fact, due to the differences in structures and functions of local tissues and cells, the diffusion of water molecules *in vivo* is often a non-normal distribution. DKI is based on the non-normal distribution diffusion of water molecules *in vivo*, and the parameters of DKI measurements include the S, K and D values. DKI is still largely in the research phase, but this technique is being explored in wider clinical studies primarily focused on central nervous system diseases[75-83]. It is encouraging that some investigators have recently begun to use DKI in experiments to investigate its applications in liver diseases both *in vitro* and *in vivo*. Rosenkrantz *et al*[84] performed DKI and DWI scans on *in vitro* samples from twelve HCC cases and showed that the DKI model may provide additional value in characterizing HCC compared to a standard monoexponential model of DWI. Filli *et al*[85] also found that whole-body DKI is technically feasible and may reflect the tissue microstructure more meaningfully than whole-body DWI. Goshima *et al*[86] studied sixty-two consecutive patients with HCC to compare the use of DKI and conventional DWI in assessing the response to treatment. They found that compared to the non-viable group, the mean kurtosis (MK) value and mean ADC value in the viable group were significantly higher and lower, respectively. The sensitivity, specificity and AUC of the ROC curve for the assessment of HCC viability were greater using MK compared to ADC. These results indicated that DKI can be a new option for assessing the post-therapeutic response in HCC.

The results described above indicate that in the near future, DKI will play an important role in evaluating the response of liver cancer to TACE treatment.

MAGNETIC RESONANCE SPECTROSCOPY

Based on chemical shift effects and MRI principles, MRS uses a Fourier transform to process free induction decay signals to convert them into spectra with distributed frequencies. The areas under different metabolic peaks along the MRS frequency axis reflect the different concentrations of different compounds and can be quantitatively measured and analyzed. Thus, MRS not only truly reflects the molecular and chemical compositions of a tissue but also indirectly depicts the metabolism in the tissue[87].

The commonly used nuclei in MRS measurements of the liver are mainly ^{31}P and ^{1}H. By using ^{1}H-MRS to determine the amounts and ratios of choline and its derivatives after TACE treatment for liver cancer, it is possible to know whether HCC survives or relapses after TACE therapy. Studies have shown significant decreases in the choline/lipid values and the absolute value of choline complexes after TACE treatment for liver cancer. Kuo *et al*[88] investigated the use of proton MRS to assess hepatic lesions *in vivo* and the use of a 3.0-T scanner to measure the changes in metabolites related to HCC after TACE treatment. Their study included forty-three consecutive patients with hepatic tumors. Among the patients with proven HCC, eight lesions were evaluated before TACE and two to five days after TACE. A significant difference was achieved in the mean choline/lipid ratio between the malignant and benign tumors, and the mean choline/lipid ratios were significantly decreased after TACE. Wu *et al*[89] also reached similar conclusions. Taken together, those studies indicated that MRS has the potential for use in the detection of early metabolite changes in HCC after TACE[90-92].

The shortcomings of MRS mainly include its rather low sensitivity and specificity in differentiating between small nodules in the liver.

MAGNETIC RESONANCE PERFUSION-WEIGHTED IMAGING

MR PWI is an MRI technology that can reflect the microvascular distribution and blood perfusion in tissues. In MR PWI, a contrast agent is intravenously injected to increase the magnetic sensitivity of local capillaries and induce local magnetic field changes, leading to reduced signals derived from shortened transverse relaxation time by proton spin dephasing in tissues. The fast scanning imaging sequence generates a series of dynamic images; based on these images, the changes in signal intensity of the contrast agent when passing the hepatic parenchyma over time are used to generate the time-intensity curve (TIC), and semi-quantitative parameters such as maximal enhancement (MaxEn), initial enhancement rate (ER)

and initial area under the curve are calculated to indirectly reflect the vascularity and perfusion in the tumor[93]. Quantitative indicators calculated using the Tofts model include K (trans), k (ep) and v (e)[94,95]. The commonly used PWI sequences include enhanced spin labeling MRI (arterial spin labeling, ASL), T2*-weighted contrast-enhanced dynamic magnetic susceptibility MRI (dynamic susceptibility contrast, DSC) and T1-weighted dynamic contrast-enhanced MRI (dynamic contrast enhancement, DCE). ASL-MRI and DSC-MRI have been mostly used in PWI studies of the brain, whereas the DCE-MRI sequence has been frequently used in PWI studies of the liver[93]. PWI is a new technology that can improve the sensitivity and specificity of liver disease diagnoses. With its high temporal and spatial resolutions, PWI can directly reflect the blood perfusion of the subject tissue and indirectly reflect the tissue's microvascular distribution. This technique better displays the lesions and tremendously helps facilitate the analysis of the disease, the identification of benign and malignant lesions and the assessment of the response to TACE treatment[96-102]. Xu *et al*[96] investigated the value of perfusion-weighted MRI in the evaluation of the intranodular hemodynamic characteristics of dysplastic nodules (DNs) and HCCs in an experimental rat model. A total of 40 rats with chemically induced DNs and HCCs were investigated. Time to peak (Tp), maximal relative signal enhancement (REmax) and the initial slope of the signal intensity (SI) *vs* the time curves of the nodules and cirrhotic liver tissues were evaluated. The nodules that precisely corresponded to the MRI were examined histologically, and the results showed that HCCs had a significantly higher REmax, a shorter Tp and a higher slope than the adjacent cirrhotic liver. The REmax and slope of DNs were significantly lower than the adjacent cirrhotic liver parenchyma. Chen *et al*[97] performed MR PWI scans in thirty-five cases of HCC 24-48 h before and 48-168 h after TACE treatment, finding that in thirty-four of the HCC patients, the time-signal intensity curve (TSC) before TACE quickly decreased and then slowly increased in the tumor region of interest. After TACE, the fluctuating range of the TSC was significantly reduced in thirty-one patients, slightly reduced in three and not significantly changed in one. These results show that MR PWI is highly useful in the clinical evaluation of the efficacy of TACE in treating HCC.

Compared with CTPI, MR PWI has a higher temporal resolution, requires a smaller dose of contrast agent and poses no risk of radiation injury. However, this spectroscopic imaging technique requires more specialized equipment, has a longer imaging time and is thus more affected by environmental factors (*e.g.*, respiratory motion and the shifts caused by respiratory motion of the target lesions, *etc.*). Moreover, the heavy load on data processing has limited the clinical application and promotion of PWI.

BLOOD OXYGEN LEVEL DEPENDENT MRI

Ogawa *et al*[103] believed that paramagnetic deoxyhemoglobin could be used as a natural contrast agent in MRI scans and that deoxyhemoglobin might have contrast effects that could be observed using a gradient echo sequence in high-magnetic field. Based on these effects, these authors investigated whether determining the microvascular blood oxygen content could be used to reflect the dynamics and pathophysiology of blood flow in organs and tissues[103,104]. *In vivo*, paramagnetic deoxyhemoglobin forms a small magnetic field and a magnetic field gradient in its surroundings, causing heterogeneity in the magnetic field within the local tissue and shortening the T2*-weighted signal. The blood oxyhemoglobin and deoxyhemoglobin have opposite magnetic properties, and when the blood flow in the local tissue and the relative amount of oxygenated hemoglobin increase, the T2*-shortening effect of deoxygenated hemoglobin weakens, causing elongated local T2*, and vice versa. Because the value of the transverse relaxation rate (R2*) and the deoxyhemoglobin concentration in the tissue are correlated, in practice, the R2* value is used as an indicator for the quantitative evaluation of changes in the oxygen content in the local tissue.

The BOLD MRI technique, which has been successfully applied in studies of the central nervous system and urogenital system[105-112], is still in the exploratory stage for the diagnosis and treatment of liver cancer. Choi *et al*[113] studied the feasibility of using carbogen-challenge BOLD MRI to assess the early response of liver tumors to chemoembolization in a rat hepatoma model. Their results demonstrated that there was a significant difference between the pre-chemoembolization and post-chemoembolization percentages by which the R2* values of the tumors changed. Zhang *et al*[114] evaluated the feasibility of performing carbogen gas-challenge BOLD MRI measurements in patients with HCC and found that in two cases, the R2* values were significantly decreased one day after TACE. These findings indicated the feasibility of using BOLD MRI to evaluate the response of liver cancer to TACE treatment.

The shortcomings of BOLD MRI are that the technology is susceptible to influences of plasma proteins, molecular diffusion, pH, temperature, pixels, blood flow and vascular course. The iron stored in the liver may also affect the results. Plotting the ROI and analyzing the R2* value are susceptible to the influences of factors such as partial volume effects, blood vessels, necrosis, and bleeding. In future research, BOLD MRI technology will play an important role in evaluating the use of TACE to treat liver cancer.

PET/CT AND PET/MRI

PET/CT can reveal the metabolic information of

tumor tissues at the molecular level and can be used to diagnose malignant cancer with high sensitivity and specificity. Fluoro-deoxy-glucose (18F-FDG) is a glucose analogue that can reflect glucose metabolism in the tissue. Because changes in tissue metabolism always precede changes in tissue structure, PET/CT can be used to assess the early response after TACE treatment and to show residual, recurring and metastasized lesions by quantitatively analyzing the changes in the standardized uptake value (SUV) of the HCC lesions before and after TACE treatment[115-120]. Kim *et al*[115] performed PET/CT and enhanced CT scans on thirty-eight liver cancer lesions in thirty-six patients after TACE therapy and found that for the viable residual lesions, the diagnostic sensitivities of PET/CT and contrast-enhanced CT in the early postembolic period were 100% and 94%, respectively; in the late postembolic period, these values were 93% and 79%, respectively. When the multiphasic CT was normal, the 18F-FDG PET/CT could clearly reveal intrahepatic tumor recurrence and/or extrahepatic metastases in patients with elevated AFP after TACE treatment for HCC[119,120].

Due to the high cost of PET/CT examination and the high radiation dose, this method is not suitable for use in the routine evaluation of TACE treatment.

PET/MRI combines the advantages of PET and MRI. This method not only provides a better soft tissue contrast than PET/CT, thus providing richer information on molecular function and form, but also overcomes the body damage caused by CT irradiation during a PET/CT examination and the false positives found in PET/CT images. Although PET/MRI is a very recent technology, preliminary studies have already shown that PET/MRI has a great potential for applications in the nervous system, cardiovascular system and neoplastic diseases[121-125]. Yu *et al*[126] reported that the additional value of functional MRI techniques in combination with PET must be considered; MR DWI, for example, has been demonstrated to significantly improve the detection of sub-centimeter sized intrahepatic HCC metastases compared with conventional liver MRI alone (84% *vs* 69%). Tsouana *et al*[127] employed hybrid 18F-Fluroethyl-Choline (FEC) PET)/MRI to evaluate the treatment response of four cases of intracranial non-germinomatous germ cell tumors, and the results showed that in two patients, faint or absent choline avidity correlated with negative histology, whereas in two other patients, persistent choline avidity in the residual mass suggested the presence of a viable tumor, which was subsequently confirmed histologically.

Currently, the use of PET/MRI to evaluate interventional treatment for HCC has been rarely reported. Fowler *et al*[128] studied the relationship between dose deposition measured by PET/MRI and the response of individual lesions to radioembolization with 90Y microspheres. Twenty-six patients undergoing lobar treatment with 90Y microspheres underwent PET/MRI within 66 h of treatment and had follow-up imaging available. The results showed that the average dose could be used to predict the responses of responders and non-responders for all lesion types. PET/MRI of the 90Y microsphere distribution in patients with colorectal metastases showed significantly higher dose volume histograms (DVHs) values for responders than non-responders. A DVH analysis of the 90Y microsphere distribution following treatment may be an important predictor of response and could be used to guide future adaptive therapy trials. With the development of PET/MRI, this technology will provide more useful information for the evaluation of interventional liver cancer treatments.

CONCLUSION

In recent years, emerging imaging techniques such as new functional imaging have been effectively used to evaluate the early response of HCC to TACE treatment. Because different imaging techniques have their own advantages and disadvantages, to detect cancer lesions as early as possible and to provide accurate information regarding the diagnosis, staging and treatment evaluation, clinical applications should combine multiple imaging techniques according to the specific circumstances such that the advantages of each technique can compensate for the shortcomings of other techniques, thereby providing a comprehensive evaluation of the lesion[129,130]. With the rapid development of medical imaging, imaging technology will play an increasingly important role in cancer diagnosis and the evaluation of the treatment response.

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ORIGINAL ARTICLE

Basic Study

Immunological changes in different patient populations with chronic hepatitis C virus infection

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Data sharing statement: No additional data are available.

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Abstract

AIM: To investigate killer inhibitory and activating receptor expression by natural killer (NK), natural killer T-like (NKT-like) and CD8+ T lymphocytes in patients with chronic hepatitis C virus (HCV) infection with elevated and with persistently normal alanine aminotransferase (PNALT).

METHODS: The percentage of peripheral blood Treg cells, KIR2DL3, ILT-2, KIR3DL1, CD160, NKG2D, NKG2C expressing NK, T and NKT-like cells, cytokine production and NK cytotoxicity were determined by flow cytometry. Twenty-one patients with chronic HCV infection with elevated alanine aminotransferase, 11 HCV carriers with persistently normal alanine aminotransferase and 15 healthy volunteers were enrolled.

RESULTS: No significant differences were observed in the percentage of total T, NK or NKT-like cells between study groups. Comparing the activating and inhibitory

receptor expression by NK cells obtained from HCV carriers with PNALT and chronic HCV hepatitis patients with elevated alanine aminotransferase, NKG2D activating receptor expression was the only receptor showing a significant difference. NKG2D expression of NK cells was significantly lower in patients with elevated alanine aminotransferase. The expression of CD160, NKG2D and NKG2C activating receptor by CD8+ T cells were significantly lower in patients with chronic HCV hepatitis than in healthy controls and in HCV carriers with PNALT. Plasma TGF-β1 levels inversely correlated with NKG2D expression by NK cells. In vitroTGF-β1 treatment inhibited NK cells cytotoxic activity and downregulated NKG2D expression. CD8+ T cells from HCV carriers with PNALT showed significantly elevated expression of CD160, NKG2D and NKG2C activating receptors compared to chronic HCV patients with elevated alanine aminotransferase. Enhanced expression of inhibitory KIR2DL3 receptor, and decreased ILT-2 expression on NK cells were also found in chronic hepatitis C patients compared to healthy controls.

CONCLUSION: Our study demonstrated a complex dysregulation of activating and inhibitory receptor expression, such as decreased NKG2D and CD160 activating receptor expression and increased KIR2DL3 inhibitory receptor expression by NK and cytotoxic T cells and may provide further mechanism contributing to defective cellular immune functions in chronic hepatitis C. Increased NKG2D receptor expression in HCV patients with persistently normal ALT suggests an important pathway for sustaining NK and CD8 T cell function and a protective role against disease progression.

Key words: Hepatitis C; Natural killer cell; NKG2D; Cytotoxicity; Cytokine

Core tip: The host immune response to hepatitis C virus (HCV) involves both innate and adaptive arms of the immune system. Natural killer (NK) cells are key components of the innate antiviral immune response. To better characterize the immune defects underlying chronic viral persistence, we focus our analysis on killer inhibitory and activating receptor expression in patients with chronic HCV infection with elevated alanine aminotransferase (ALT) and also in patients with HCV carriers with persistently normal ALT. Decreased NKG2D and CD160 activating receptor expression and increased KIR2DL3 inhibitory receptor expression by NK and cytotoxic T cells in patients with chronic hepatitis C contributing to defective cellular immune functions.

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INTRODUCTION

More than 170 million people worldwide are chronically infected by hepatitis C virus (HCV)[1]. Approximately 20% of HCV infected patients resolves acute hepatitis and clears the virus, but most develop life-long infection, making HCV a leading cause of chronic liver disease, cirrhosis and hepatocellular carcinoma[2]. No vaccine is currently available to prevent hepatitis C[3]. The mechanisms favoring persistent infection are still poorly understood.

Approximately 30% of patients with chronic HCV (CHC) infection show persistently normal alanine aminotransferase (ALT) levels and are considered CHC carriers[4].

The host immune response to HCV involves both innate and adaptive arms of the immune system. Natural killer (NK) cells are key components of the innate antiviral immune response. NK cells, a subset of lymphocytes, represent between 5% and 15% of mononuclear cells in the peripheral blood and up to 45% in some organs, such as the liver. The major functional role of NK cells is the defense against tumor cells and lyses of virus-infected cells[5]. However, given their potent cytotoxic and cytokine secretion potential, their activity needs to be tightly regulated. The activation state of an NK cell is determined partly by the integration of activating and inhibitory signals after interaction of surface NK cell receptors, including the highly diverse killer Ig-like receptor (KIR) family, with ligands found on target cells.

Previous results from our laboratory showed an impaired NK activity in chronic hepatitis[6]. Therefore, altered function of NK cells might be one of the mechanisms by which viruses escape the immune system. Activating receptors on NK and T cells might provide not only the machinery to induce proliferation and fight off infection, but also to support maintenance of the cells critically needed under conditions of extended viral infections during CHC.

There is new evidence that NK cells also can be activated to negatively regulate T cell responses because they can produce interleukin-10 (IL-10)[7,8]. Under conditions of continued stimulation - like CHC infection - IL-10 response by NK cells could limit the magnitude of CD8 T cell response and protect from T cell-mediated disease. If the NK cells are not regulated properly, the regulation is lost with detrimental consequences.

Natural killer T-like (NKT-like) cells are a sublineage of T cells that share characteristics of conventional T cells and NK cells and bridge innate and adaptive immunity[9]. The most characteristic immunoregulatory function

of NKT cells is their ability to promptly secrete large amounts of Th1 and Th2 cytokines including interferon-γ (IFN-γ) and IL-4, respectively, upon stimulation[10]. Downstream, this culminates in the activation of different cell types of the innate immune system such as macrophages, NK cells, and dendritic cells as well as effector T cells of the adaptive immune system.

Although shown to mediate immunity against a wide range of pathogenic microbes, including bacteria, fungi, parasites, and viruses, the mechanism(s) by which NKT cells are activated during infection is still unclear. NKT-like cells are abundant in the liver; however, their role in the control of hepatitis C virus infection remains to be determined[11].

CD8+ T cells are important in viral elimination by using direct killing of infected cells and non-cytotoxic mechanisms such as the secretion of antiviral cytokines [IFN-γ or tumor necrosis factor (TNF)-α][12]. Despite the detection of HCV-specific CD8+ T cells in the peripheral blood and the intrahepatic lymphocytic infiltrate in patients with chronic hepatitis C, the virus can persist. This persistence in spite of the presence of these cytotoxic cells is still unexplained and suggests that cell killing is not sufficient to eliminate the virus. Studies in humans have revealed that even strong CD8+ T cell responses in the acute phase of infection may not be adequate to prevent progression to chronicity[13-15]. Several investigators have clearly shown that HCV-specific CD8+ T cells have functional defects during chronic infection, as indicated by impaired IFN-γ production, cytotoxic effector functions, and *in vitro* proliferation[16,17]. While the mechanisms responsible for the dysfunctions of HCV-specific T cells in chronically infected patients remain unclear, recent studies suggest a major contribution of regulatory T cells.

To better characterize the immune defects underlying chronic viral persistence, in this study we focus our analysis on killer inhibitory and activating receptor expression in patients with chronic hepatitis C virus infection with elevated ALT and also in patients with CHC carriers with persistently normal ALT (PNALT) by NK, NKT-like and CD8+ T lymphocytes, given the central role played by these cells in the control of viral infections. Progress in the understanding of antiviral immune responses in CHC carriers with PNALT could elucidate key mechanisms playing a role in the control of viral infection.

MATERIALS AND METHODS

Patients

Persistently normal ALT was defined as ALT < 30 IU/L in men, ALT < 19 IU/L in women measured every 3 mo over an 18-mo period. Patients with Fibroscan result suggesting > F1 liver fibrosis (LS > 7.0 kPa) were excluded from the CHC with PNALT group. Eleven age-matched healthy blood donors served as controls.

All HCV subjects were seronegative for anti-HIV 1, 2 antibodies (ELISA 2.0, Abbott, Wiesbaden, Germany), and HBsAg (Hepanostica Uniform II, Organon Teknika, Oss, The Netherlands), and were positive for both anti-HCV antibody and HCV-RNA. Diagnosis of chronic hepatitis C was established by means of histology in all symptomatic patients, but liver biopsy was not performed in CHC carriers with PNALT.

HCV markers

Anti-HCV antibody was examined using enzyme-linked immunoabsorbent assay (ELISA) (Detect-HCV Ab, Biochem Immunosystem, ITC, Canada). Serum HCV RNA detection and quantification were performed with Roche Cobas Amplicor HCV 2.0 assay (lower limit of detection < 50 IU/mL) and Cobas Amplicor HCV Monitor Assay (Roche Diagnostics) according to the manufacturer's instructions.

Sample preparation

Venous blood samples were collected in heparanized tubes and peripheral blood mononuclear cells (PBMC) were prepared by Ficoll-Paque density gradient centrifugation.

Antibodies and flow cytometry

Separated cells were washed in PBS and incubated for 30 min at room temperature with the monoclonal antibodies. The following monoclonal antibodies were used for these studies: FITC-conjugated anti-CD3, anti-CD8, anti-CD4, PE-conjugated anti-CD25, anti-KIR2DL3 (CD158b), anti-ILT-2 (CD85), anti-NKG2C, anti-CD160, anti-NKG2D, anti-KIR3DL1 (CD158e) and APC-conjugated anti-CD56. After washing the cells in PBS, cells were fixed with 4% paraformaldehyde, stored at 4 ℃, in dark, to be processed for FACS analysis. At least 10000 cells were analyzed on the FACS Calibur flow-cytometer (Becton Dickinson Immunocytometry Systems, Erembodegen, Belgium) after single gating on lymphoid cells for all mAb combinations. The percentage of positive cells was calculated using Cellquest software (Becton Dickinson, San Diego, CA, United States). Figure 1 shows the gating technique used to detect different lymphocyte subpopulations with representative flow cytometric dot plots. The effect of TGF-β1 treatment on NKG2D, CD160 and KIR2DL3 expression by NK cells.

PBMC were separated from heparinized venous blood on Ficoll-Paque gradient. One million cells were treated with recombinant active TGF-β1 protein (1 ng/mL) for 48 h at 37 ℃ in a tissue culture incubator. NKG2D, CD160 and KIR2DL3 expression by NK cells was determined by flow cytometry.

NK and CD8+ T separation and cytometric bead array

Natural killer and CD8+ T cells were separated by MACS Cell Separation Technology (all reagents and instruments from Miltenyi Biotec, Frank Diagnosztika Kft., Budapest, Hungary). PBMCs were first magnetically labeled with CD56 or CD8 MicroBeads

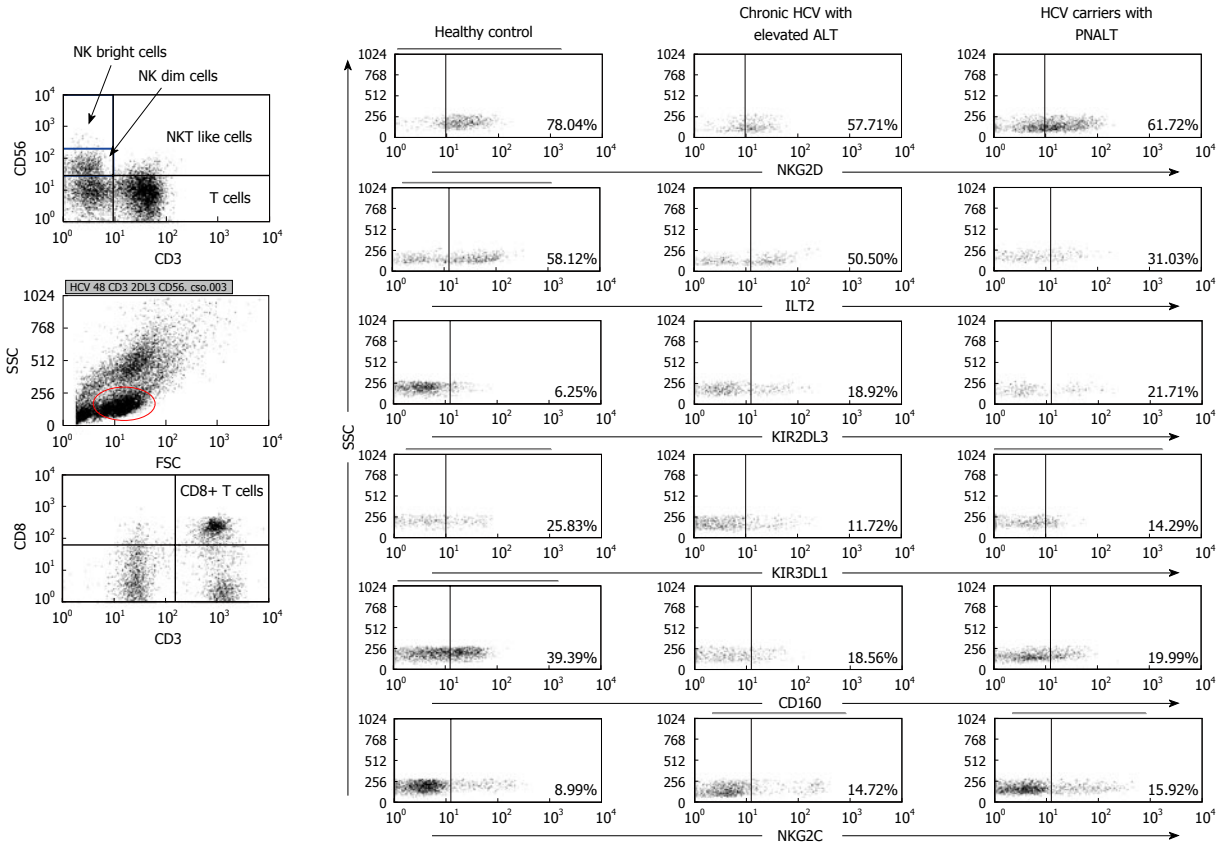

Figure 1 Gating strategy and representative flow cytometric dot plots. Figure 1 shows the gating technique used to detect different lymphocyte subpopulations. For analysis of NK, NK^{dim}, NK^{bright}, NKT-like and CD8+ T cells cells lymphogate was created based on physical characteristics typical of lymphoid cells using forward and side scatter parameters. Representative dot plots show the expression of NKG2D, ILT-2, KIR2DL3, KIR3DL1, CD160 and NKG2C by NK cells in the peripheral blood from patients with chronic HCV with elevated ALT or PNALT and in healthy individuals. HCV: Hepatitis C virus; ALT: Alanine aminotransferase; PNALT: Persistently normal ALT; NK: Natural killer cell.

according to the manufacturer's instructions and CD56+ or CD8+ T cells were positively selected on the cell separation column. In the next step, the magnetic beads bound to the cell surface were enzymatically released from the CD56+ cells, which were then magnetically labeled with CD3 MicroBeads and the CD3+ subpopulation positively selected to compose the CD3+CD56+ T cell population. The remaining fraction of the CD56+ cells, which did not bind the CD3 beads composed the CD3-CD56+ NK cell population. Purity was greater than 95%. CD8+ and CD3-CD56+ cell populations were stimulated with 1 μg/mL of ionomycin and 25 ng/mL of PMA (Sigma-Aldrich, Sigma-Aldrich Kft., Budapest, Hungary) in RPMI 1640 Medium containing 10% fetal bovine serum, penicillin and streptomycin (all from Invitrogen, Csertex Kft., Budapest, Hungary) overnight for cytokine production. The levels of IL-2, IL-4, IL-5, IL-10, IFN-γ and TNF-α were determined from the culture supernatants with cytometric bead array (CBA) (#550749, BD Biosciences, Soft Flow Hungary Kft., Pécs, Hungary) using different capture beads according to the manufacturer's instructions to detect the respective cytokines. Samples were analyzed right after the experiment on a FACS calibur flow cytometer (BD Immunocytometry Systems, Erembodegen, Belgium) calculating the amount of cytokines with CBA Software (BD Biosciences, San Diego, CA, United States).

Cytotoxic assay for NK cell activity

Cytotoxicity was determined as described earlier[18]. Cytotoxic activity of NK cells was evaluated by FACS analysis. Target cells (1×10^6) were pre-stained with the green fluorescent membrane dye PKH67 (Sigma, Hungary) and effector cells were added to 25×10^4 target cells to yield effector to target (E:T) ratios of 12.5:1, 25:1, and 50:1. The tubes were centrifuged for 30 s at 1200 rpm to pellet the effector and target cells together. These cell mixtures were incubated for 4 h at 37 ℃ in 5% CO_2. After incubation the cell mixture was centrifuged at 1200 rpm and stained with propidium iodide (PI, 5 μg/mL, Sigma, Hungary). Dead target cells were identified by simultaneous PKH67 and PI-positivity. Target cells incubated without effector cells were used to assess spontaneous cell death. The percentage of lysed target cells was calculated by subtracting background (spontaneous cell death) expression from experimental samples. Cytotoxicity was expressed as the percentage of lysed target cells in each effector-to-target ratio.

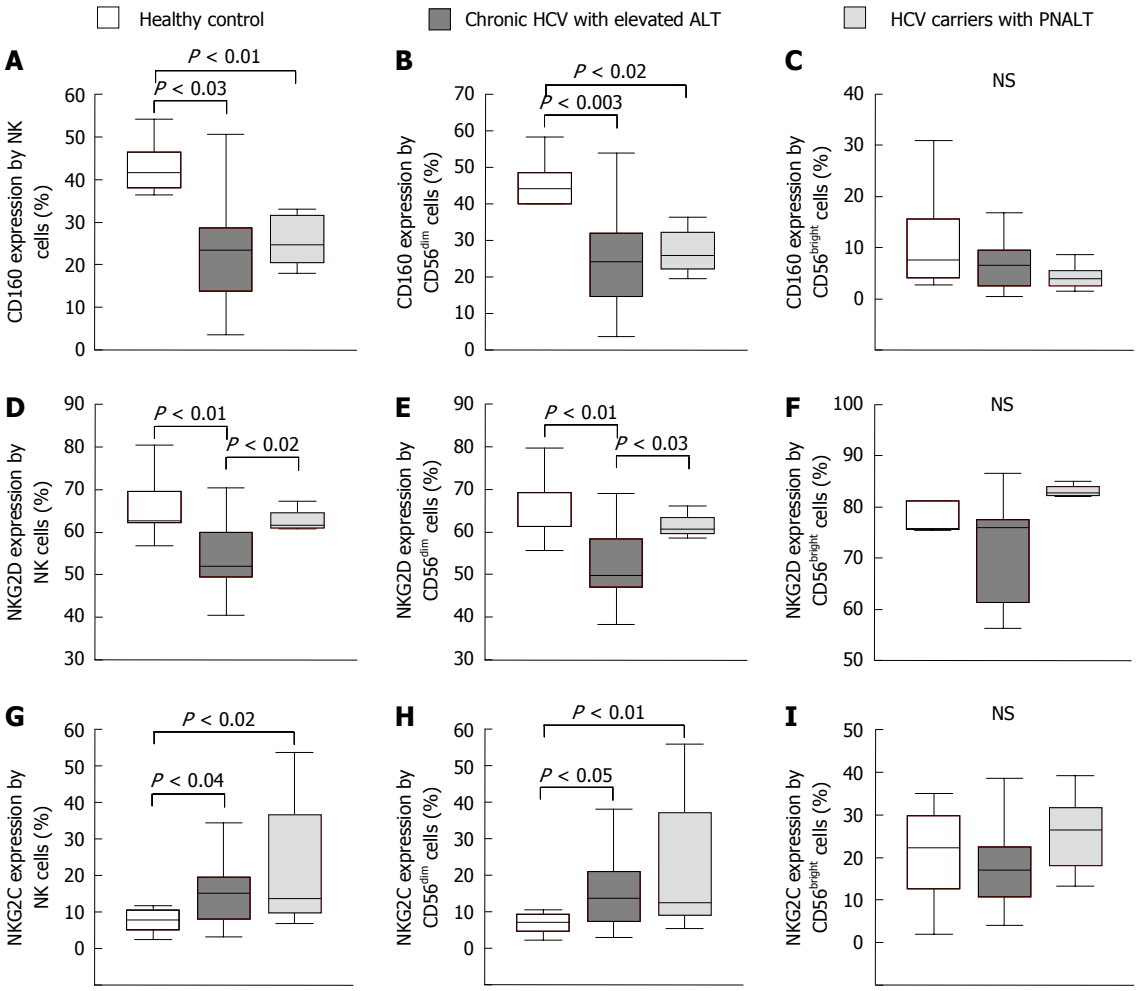

Figure 2 Activating natural killer cell receptor expression by natural killer cells in the peripheral blood from patients with chronic hepatitis C virus with elevated alanine aminotransferase or persistently normal alanine aminotransferase and in healthy individuals. The expression of CD160 (A-C), NKG2D (D-F) and NKG2C (G-I) by NK cells and NK cell subsets in the peripheral blood from patients with chronic HCV with elevated ALT or PNALT and in healthy individuals. The solid bars represent medians; the boxes indicate the interquartile ranges and the lines show the most extreme observations. Differences were considered statistically significant for *P* values ≤ 0.05. HCV: Hepatitis C virus; ALT: Alanine aminotransferase; PNALT: Persistently normal ALT; NK: Natural killer cell.

Effect of TGF-β1 on NK cytotoxicity

PBMC were separated from heparinized venous blood on Ficoll-Paque gradient. One million cells were treated with recombinant active TGF-β1 protein (1 ng/mL) for 48 h at 37 ℃. Following incubation we determined cytotoxicity of NK cells by flow cytometry.

Statistical analysis

Statistical analysis was performed using non-parametric Mann-Whitney *U*-test with statistical software SPSS version 11.0 package (SPSS, Inc. Chicago, IL, United States) (Figures 2-5). Results are expressed as mean value ± standard error of the mean (SEM). Statistical comparisons were made by using one-way ANOVA with Bonferroni correction (Figures 6 and 7). The results were expressed as the mean value ± SEM. Differences were considered significant if the *P* value was equal to or less than 0.05. Correlation between variables was assessed by calculating Spearman rank correlation coefficient. Differences were accepted as significant at a level of $P < 0.05$.

RESULTS

Patients

Twenty one patients with CHC infection with elevated ALT > 2 × ULN (11 males, 10 females, mean age: 57 years; range 38-70 years) and 11 (2 males, 9 females, mean age: 56 years; range 41-63 years) HCV carriers with persistently normal ALT were studied.

Lymphocyte frequency

No significant differences were observed in the percentage of helper (CD4+) or cytotoxic (CD8+) T cells, regulatory (CD4+CD25^{high}) T cells, NK (CD3-CD56+) or NKT-like (CD3+CD56+) cells in peripheral blood of patients with CHC hepatitis with elevated ALT compared to CHC patients with PNALT and also to

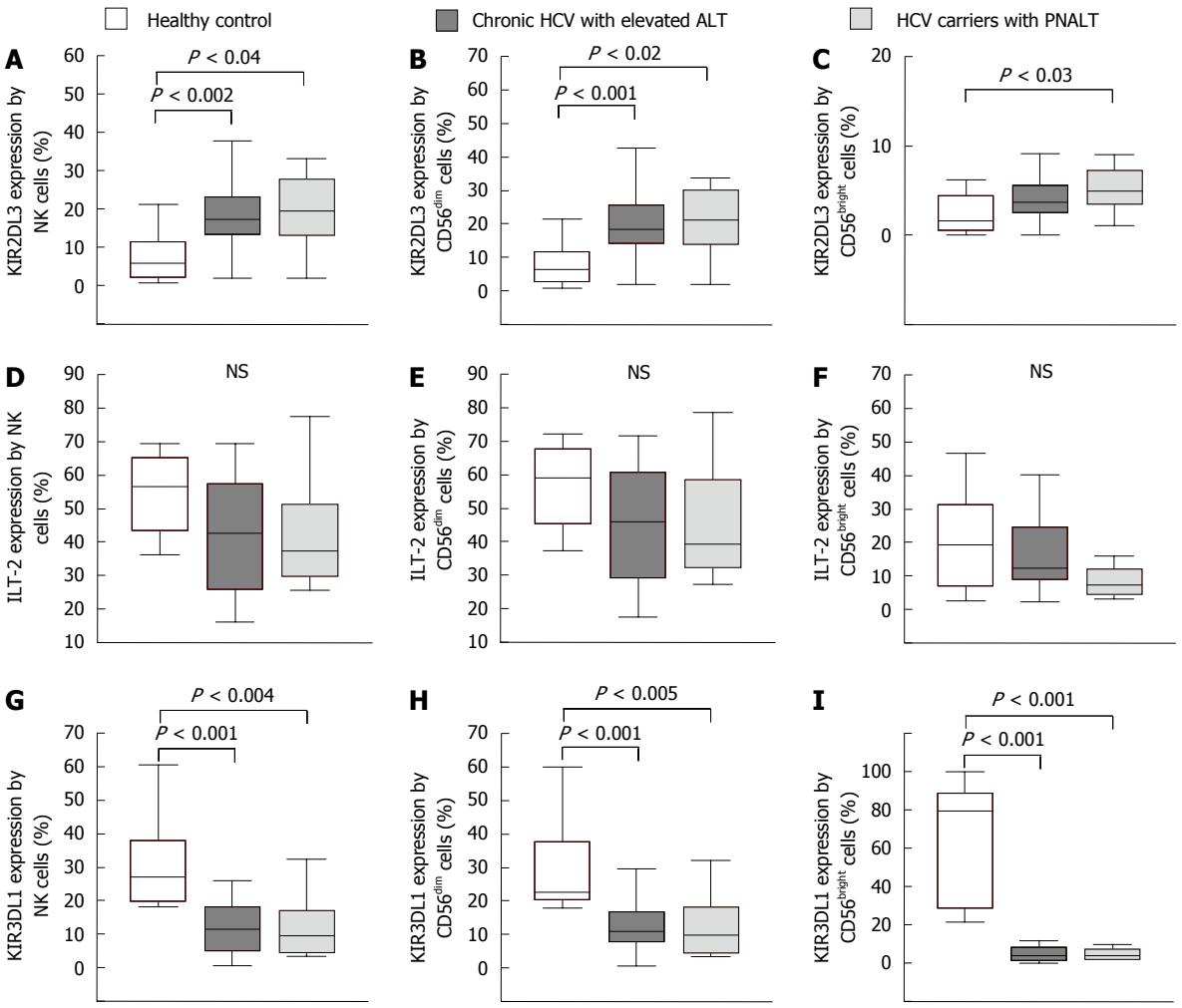

Figure 3 Inhibitory natural killer cell receptor expression by natural killer cells in the peripheral blood from patients with chronic hepatitis C virus with elevated alanine aminotransferase or persistently normal alanine aminotransferase and in healthy individuals. The expression of KIR2DL3 (A-C), ILT-2 (D-F) and KIR3DL1 (G-I) by NK cells and NK cell subsets in the peripheral blood from patients with chronic HCV with elevated ALT or PNALT and in healthy individuals. The solid bars represent medians; the boxes indicate the interquartile ranges and the lines show the most extreme observations. Differences were considered statistically significant for *P* values ≤ 0.05. HCV: Hepatitis C virus; ALT: Alanine aminotransferase; PNALT: Persistently normal ALT; NK: Natural killer cell.

healthy controls (Table 1).

Activating and inhibitory NK cell receptor expression by NK cells in the peripheral blood from patients with CHC with elevated ALT or PNALT and in healthy individuals

The phenotypes and functional activities of various populations of innate effectors have been reported to be impaired in all stages of HCV infection[6,19-21]. The balance of activating and inhibitory signals through the killer activating and the inhibitory receptors control NK cell activity. Since the role of NK cells determining disease inflammatory activity reflected by ALT elevation in chronic hepatitis C is not clear, we investigated different activating and inhibitory NK cell receptor expression by NK cells in patients with CHC with elevated ALT or PNALT.

CD160 and NKG2D activating NK cell receptor expression was significantly lower in patients with CHC infection than in healthy controls (Figure 2A and D). NKG2D expression by NK cells was significantly lower in CHC patients with elevated ALT than in CHC positive patients with PNALT (Figure 2D). Expression of NKG2C was higher in NK cells from patients with CHC infection as compared to healthy controls but significantly lower than CHC carriers (Figure 2G). NK cells can be divided into two populations based on the intensity of the CD56 marker at the cell surface. $CD56^{dim}$ NK cells are the more cytotoxic subset, whereas $CD56^{bright}$ cells are poorly cytotoxic and preferentially secrete cytokines when activated[22]. The majority of NK cells were of the $CD3^{-}CD56^{dim}$ phenotype and our data shows that this cell population represents the above mentioned alterations in activating receptor expression by NK cells (Figure 2B, E and H). In contrast to $CD56^{dim}$ cells, we found no significant difference in the killer activating NK cell receptor expression by $CD56^{bright}$ cells between the investigated groups (Figure 2C, F and I).

An enhanced expression of inhibitory KIR2DL3 receptor on NK cells was found in peripheral blood of patients with CHC infection and also in CHC carriers

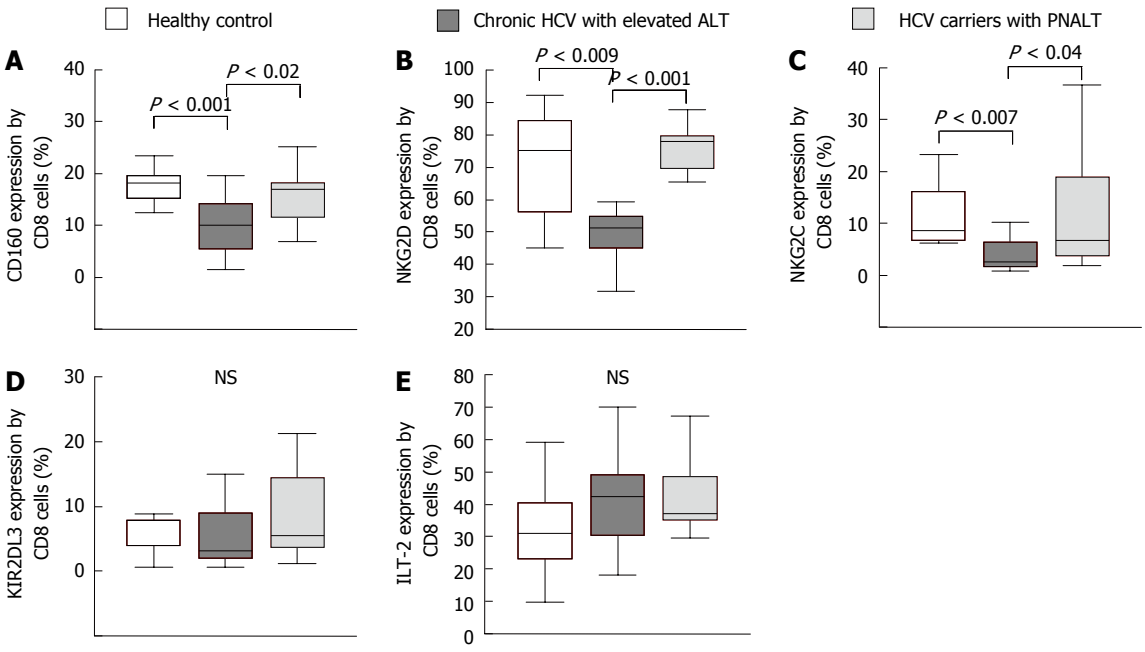

Figure 4 Activating and inhibitory natural killer cell receptor expression by CD8+ T cells in the peripheral blood from patients with chronic hepatitis C virus with elevated alanine aminotransferase or persistently normal alanine aminotransferase and in healthy individuals. The expression of CD160 (A), NKG2D (B), NKG2C (C), KIR2DL3 (D) and ILT-2 (E) by CD8+ T cells in the peripheral blood from patients with chronic HCV with elevated ALT or PNALT and in healthy individuals. The solid bars represent medians; the boxes indicate the interquartile ranges and the lines show the most extreme observations. Differences were considered statistically significant for *P* values ⩽ 0.05. HCV: Hepatitis C virus; ALT: Alanine aminotransferase; PNALT: Persistently normal ALT; NK: Natural killer cell.

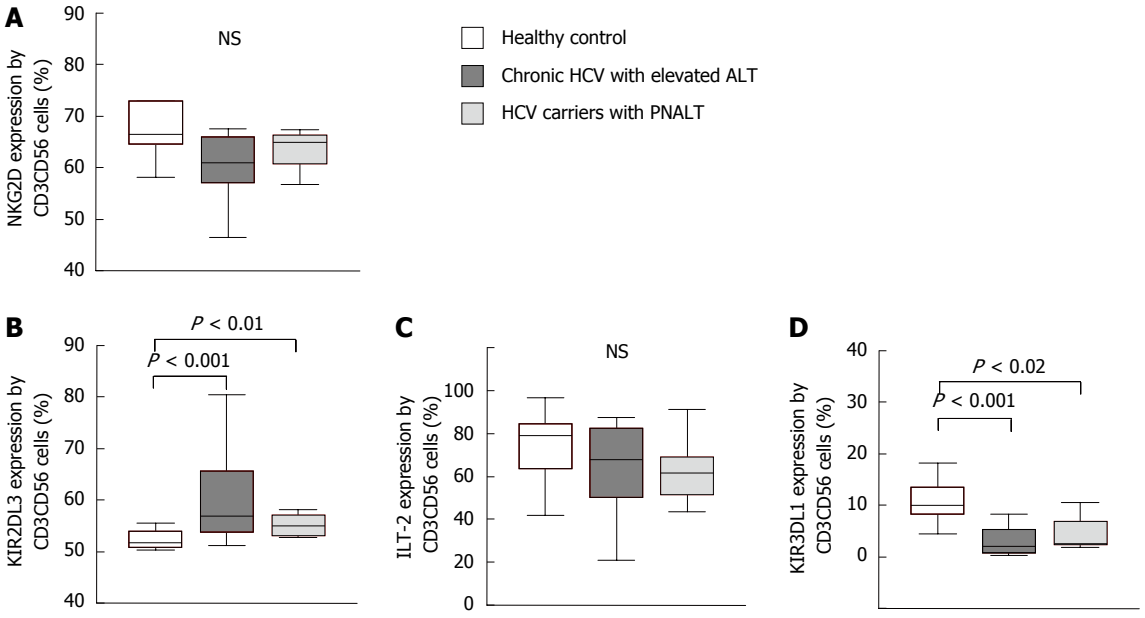

Figure 5 Activating and inhibitory natural killer cell receptor expression by NKT-like cells in the peripheral blood from patients with chronic hepatitis C virus with elevated alanine aminotransferase or persistently normal alanine aminotransferase and in healthy individuals. The expression of NKG2D (A), KIR2DL3 (B), ILT-2 (C) and KIR3DL1 (D) by NKT-like cells in the peripheral blood from patients with chronic HCV with elevated ALT or PNALT and in healthy individuals. The solid bars represent medians; the boxes indicate the interquartile ranges and the lines show the most extreme observations. Differences were considered statistically significant for *P* values ⩽ 0.05. HCV: Hepatitis C virus; ALT: Alanine aminotransferase; PNALT: Persistently normal ALT; NK: Natural killer cell.

with PNALT in comparison to healthy controls (Figure 3A). No significant differences were found between study groups in regarding expression of ILT-2 on NK cells (Figure 3D-F). KIR3DL1 inhibitory NK cell receptor expression was significantly decreased by NK, $CD56^{dim}$ and $CD56^{bright}$ cells in patients with CHC irrespectively of ALT compared to healthy individuals (Figure 3G-I).

Activating and inhibitory receptor expression by CD8+ T cells

Significantly lower percentage of CD160, NKG2D and NKG2C activating receptor expressing CD8+ T cells

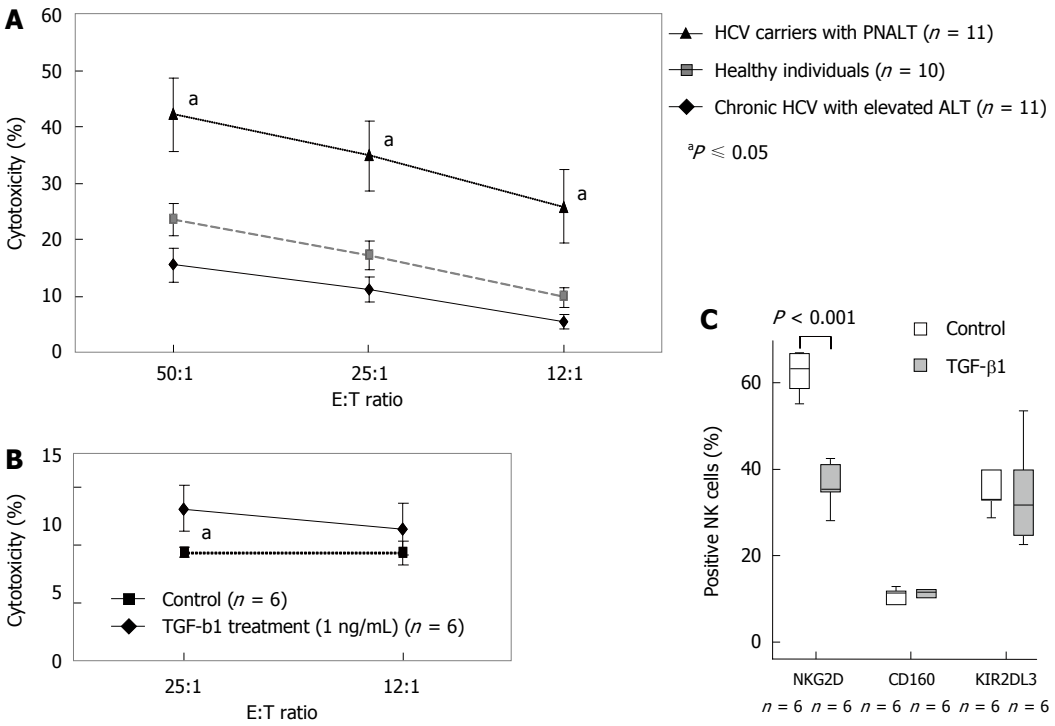

Figure 6 Natural killer cell cytotoxicity against K562 cells in patients with chronic hepatitis C virus with elevated alanine aminotransferase or persistently normal alanine aminotransferase and in healthy individuals and the effect of in vitro TGF-β1 treatment on the cytotoxicity and natural killer cell receptor expression of freshly isolated natural killer cells. A: Cytotoxicity of NK cells isolated from healthy individuals, HCV carriers with PNALT and chronic HCV with elevated ALT. Cytotoxic activity of NK cells as a percentage of lysed cells is indicated in patients with chronic HCV with elevated ALT or PNALT and in healthy individuals at different effector and target cell ratios. Statistical comparisons were made by using one-way ANOVA with Bonferroni correction. The results were expressed as the mean value ± standard error of the mean (SEM). $^aP \leq 0.05$, significant from patients with chronic HCV with elevated ALT and healthy individuals. B: Cytotoxicity of TGF-β treated NK cells isolated from healthy individuals. Cytotoxic activity of NK cells as a percentage of lysed cells is indicated after TGFβ1 treatment (1 ng/mL) at different effector and target cell ratios. C: Expression of NKG2D, KIR2DL3 and CD160 receptors by TGF-β-treated NK cells. Different NK cell receptor expression by NK cells after TGFβ1 treatment (1 ng/mL). Statistical comparisons were made by one-way ANOVA with Bonferroni correction. The solid bars represent medians; the boxes indicate the interquartile ranges and the lines show the most extreme observations. Differences were considered statistically significant for *P* values ≤ 0.05. E: Effector cell; T: Target cell; HCV: Hepatitis C virus; ALT: Alanine aminotransferase; PNALT: Persistently normal ALT; NK: Natural killer cell.

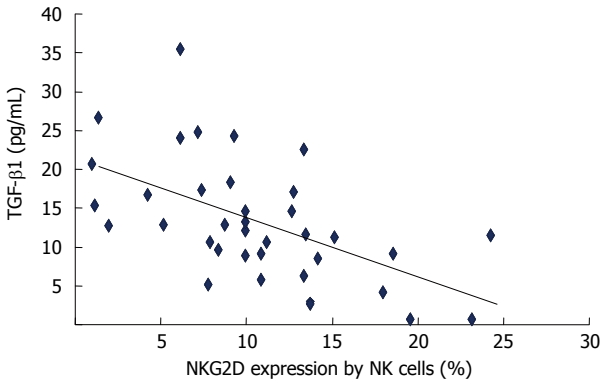

Figure 7 The correlation of plasma transforming growth factor β1 levels with NKG2D expression by natural killer cells in patients with chronic hepatitis C virus hepatitis. Shown is plasma TGF-β1 levels with NKG2D expressed by NK cells. Correlation between variables was assessed by calculating Spearman rank correlation coefficient. NK: Natural killer cell; TGF-β1: Transforming growth factor β1.

were found in patients with CHC hepatitis than in healthy controls and in CHC carriers with PNALT. (Figure 4A-C). We found no difference in the percentage of inhibitory receptor (KIR2DL3 and ILT-2) expression by CD8+ T cells in patients with CHC infection compared to healthy controls (Figure 4D and E).

Activating and inhibitory NK cell receptor expression by NKT-like cells

No significant difference was found between healthy controls and patients with HCV infection with respect to expression of NKG2D on NKT-like cells (Figure 5A). KIR2DL3 inhibitory NK cell receptor expression by NKT-like cells revealed an enhanced proportion of KIR2DL3-expressing cells in peripheral blood of patients with CHC in comparison to healthy controls (Figure 5B).

Regarding expression of ILT-2 inhibitory receptors, we found no difference in the percentage of ILT-2 receptor expressing NKT-like cells in study groups (Figure 5C). KIR3DL1 inhibitory receptor expression -similarly to NK cells- was significantly decreased on NKT-like cells in patients with CHC irrespective of ALT compared to healthy individuals (Figure 5D).

Alteration of cytokine production by NK and CD8+ T cells in CHC infection

NK cells produced significantly higher IL-10, TNF-α and IFN-γ in patients with CHC compared to healthy individuals (Table 2). In addition, IL-2, IL-4, IL-5,

Table 1 Peripheral blood mononuclear cell phenotype characteristics in patients with chronic hepatitis C virus with elevated alanine aminotransferase or persistently normal alanine aminotransferase and in healthy individuals

	Healthy individuals	Chronic HCV with elevated ALT	HCV carriers with PNALT
Percentage of PBL			
CD3+CD4+	29.38 ± 8.11	33.15 ± 9.08	34.46 ± 6.49
CD3+CD4+CD25+	4.27 ± 1.62	4.05 ± 1.57	5.24 ± 1.43
CD3+CD4+CD25^{bright}+	0.52 ± 0.26	0.38 ± 0.26	0.40 ± 0.13
CD3+CD56+	2.99 ± 1.73	5.07 ± 4.15	3.48 ± 2.72
CD3-CD56+	17.34 ± 7.42	16.42 ± 5.26	17.04 ± 7.71
CD3-CD56^{dim}+	16.04 ± 7.36	14.58 ± 5.72	15.92 ± 7.14
CD3-CD56^{bright}+	1.15 ± 0.64	1.75 ± 1.54	1.27 ± 1.01
CD3+CD8+	28.59 ± 4.55	25.27 ± 8.77	24.66 ± 6.14

Statistical comparisons were made by using the ANOVA tests. The results were expressed as the mean value ± SD. HCV: Hepatitis C virus; ALT: Alanine aminotransferase; PNALT: Persistently normal ALT; NK: Natural killer cell.

IL-10, TNF-α and IFN-γ production by NK cells isolated from CHC carriers with PNALT was significantly higher compared to healthy controls (Table 2). PNALT was associated with higher level of IL-4 and TNF-α compared to CHC hepatitis group (Table 2). CHC infection was associated with significantly higher IL-4, IL-5, IL-10 and TNF-α levels produced by CD8+ T cells compared to healthy individuals. Only IL-10 differed significantly between PNALT and elevated ALT group. CD8+ cells of CHC carriers with PNALT produced significantly higher amount of IL-10 compared to CHC hepatitis group (Table 2).

Cytotoxicity of NK cells and the effect of in vitro TGF-β1 treatment

Earlier we demonstrated that TGF-β1 levels significantly higher in patients with chronic hepatitis C with elevated ALT compared to PNALT group and healthy individuals. TGF-β1 levels positively correlated with Knodell histological activity index assessed by liver biopsy. Since impaired NK cell function has been attributed to down-modulation of activating receptors NKG2D *via* secretion of TGF-β1 in lung and colorectal cancer patients, we investigated the effect of TGF-β1 treatment on NK cell cytotoxicity[23].

To determine whether decreased NKG2D expression by NK cells in CHC patients with elevated ALT is potentially related to their increased TGF-β1 levels, we studied the in vitro effect of TGF-β1 treatment on the cytotoxicity of NK cells in response to co-culture with the classical NK cell target K562 cells. NK cells from CHC carriers with PNALT showed significantly higher cytotoxic activity compared to patients with chronic CHC with elevated ALT or healthy individuals (Figure 6A). Treatment of freshly isolated NK cells with TGF-β1 suppressed NK-dependent lysis of K562 cells (13.21 *vs* 9.43, $P < 0.01$) (Figure 6B).

Effect of TGF-β1 on NKG2D expression of freshly isolated NK cells

To investigate if TGF-β1 was responsible for down-modulation of NKG2D, we incubated freshly isolated NK cells obtained from healthy volunteers with 1 ng/mL TGF-β1 for 48 h and analyzed NKG2D expression by FACS. Incubation of NK cells with TGF-β1 significantly down-regulate surface NKG2D expression by NK cells (63.08 *vs* 36.21, $P < 0.01$). In contrast, TGF-β1 did not alter the level of other NK receptors, including the activating NK cell receptor, CD160 (37.47 *vs* 34.04 NS) or the inhibitory NK cell receptor, KIR2DL3 (11.05 *vs* 11.04 NS). These data suggest that TGF-β1 specifically down-modulates NKG2D without affecting other NK receptors.

Together, our data strongly suggest that secretion of TGF-β1 in CHC patients can down-modulate NKG2D expression by NK cells (Figure 6C). Plasma TGF-β1 levels inversely correlated with NKG2D expression by NK cells in CHC infected individuals (Figure 7).

DISCUSSION

Following acute HCV infection a majority of healthy adults will develop persistent viremia. Effective clearance of an acute viral infection typically requires the coordinated function of multiple arms of the immune system, including the innate immune system (interferons, NK and NKT-like cells), as well as the acquired immune response specific to a given pathogen (CD4+ and CD8+ T cells). A functional impairment of NK, NKT-like and CD8 T cells has been reported in CHC infections by several observations, and different mechanisms have been proposed to explain this defective function[5,19-21,24].

Natural killer cell receptors are important regulators of NK and CD8+ T cell functions. Regarding the fact that impaired activities of CD8+ T[6,20] and NK cells[19,21,24] have been reported in patients with chronic hepatitis C infection, we analyzed whether dysregulation of NK cell receptors on these cell might be involved in the inefficient cellular immune response observed in chronic hepatitis C. In this study we analyzed the expression of different activating and inhibitory NK cell receptors in CHC patients with elevated and with persistently normal ALT.

We found that the percentages of NKG2D or CD160 activating receptor positive NK and CD8+ T cells were significantly decreased in CHC patients with elevated ALT compared to healthy individuals. This discrepancy was not found in CHC infected patients with persistently normal ALT. CD8+ T cells from CHC carriers with PNALT showed significantly elevated expression of CD160, NKG2D and NKG2C activating receptors compared to CHC patients with elevated ALT. Comparing the activating and inhibitory receptor expression by NK cells obtained from CHC carriers with PNALT and CHC hepatitis patients with elevated ALT,

Table 2 Cytokine production by natural killer cell and CD8+ T cells in the peripheral blood from patients with chronic hepatitis C virus with elevated alanine aminotransferase or persistently normal alanine aminotransferase and in healthy individuals

	IL-2	IL-4	IL-5	IL-10	TNF-α	IFN-γ
NK cells						
Healthy individuals	400.4 ± 357.4	32.4 ± 23.5	6.3 ± 1.39	5.50 ± 0.23	1301.7 ± 417.3	2739.2 ± 61.2
Chronic HCV with elevated ALT	817.3 ± 316.2	20.2 ± 8.6[c]	27.9 ± 15.6	9.30 ± 1.63[a]	3034.3 ± 649.7[a,c]	3268.6 ± 140.4[a]
HCV carriers with PNALT	1182.3 ± 447.7[e]	169.5 ± 48.1[a]	18.3 ± 5.83[e]	9.70 ± 1.22[a]	4491.6 ± 148.6[b]	3231 ± 77.9[b]
CD8+						
Healthy individuals	1944.5 ± 303.9	26.1 ± 6.9	65.5 ± 20.8	18.2 ± 4.9	1369.0 ± 224.5	2608.4 ± 81.2
Chronic HCV with elevated ALT	2440.5 ± 378.3	276.3 ± 98.1[a]	461.2 ± 213.9[a]	58.3 ± 15.8[a,c]	3247.4 ± 590.8[a]	2721.9 ± 78.7
HCV carriers with PNALT	2502.1 ± 238.5[e]	253.1 ± 95.2[a]	374.8 ± 171.6[a]	258.3 ± 71.9[e]	3895.0 ± 218.3[b]	2719.7 ± 127.0

Concentrations of cytokines are given in picograms per milliliter (mean ± SD). [a]$P < 0.05$, [e]$P < 0.02$, [b]$P < 0.001$, *vs* healthy individuals, significantly different; [c]$P < 0.05$ *vs* symptomatic individuals, significantly different. HCV: Hepatitis C virus; ALT: Alanine aminotransferase; PNALT: Persistently normal ALT; NK: Natural killer cell; IL-2: Interleukin-2; TNF-α: Tumor necrosis factor α; IFN-γ: Interferon-γ.

NKG2D activating receptor expression was the only receptor showing a significant difference. Investigating $CD56^{dim}$ NK cells, NKG2D receptor expression was significantly elevated in persistently normal ALT group compared to CHC hepatitis patients.

Analyzing inhibitory NK cell receptors expressed by CD8+ T, NK or NKT-like cells, we did not find any difference in the expression of KIR2DL3, ILT-2 or KIR3DL1 between patients with CHC infection with elevated ALT and CHC carriers with PNALT. Interestingly KIR3DL1 expression by NK and NKT-like cells was significantly lower in patients with chronic hepatitis C with elevated ALT compared to healthy individuals. On the other hand KIR2DL3 expression by NK and NKT-like cells were significantly increased in patients with CHC infection with elevated ALT and CHC carriers with PNALT compared to healthy individuals.

The mechanisms by which NK and NKT-like cells in hepatitis express KIR2DL3 at considerable high levels remain elusive. One possibility is that the expression levels of NK receptors may be modified by various types of cytokines released under chronically inflamed conditions. Given previous findings that high level of serum TGF-β production were observed in CHC infected patients[25] and that TGF-β can up regulate the expression of inhibitory receptors on NK cells[26] we hypothesized that TGF-β would contribute to the high expression of KIR2DL3 on NK and NKT-like cells in CHC infection. We found that TGF-β1 significantly down-regulated surface NKG2D expression by NK cells, but did not alter the level of other NK receptors, including the activating NK cell receptor CD160 or the inhibitory NK cell receptor KIR2DL3, suggesting that other factors, including the virus itself, play a role in the modulation of activating or inhibitory receptor expression.

The activation status of NK, NKT-like and CD8+ T cells depends on the balance between activating and inhibitory signals delivered by surface receptors. Thus, our present findings of up-regulated expression of inhibitory receptors combined with the concomitant down-regulation of activating receptors synergistically leads to the dominant delivery of inhibitory signals to NK, NKT cells and KIR receptor-positive T cells. This altered receptor expression is reflected by impaired cytotoxic function of NK cells. Our data agree with other studies[27,28], which reported a significant reduction in NK cytotoxic activity in patients with CHC infection, but disagree with several other studies[24,29].

Our results show that NK cells from CHC carriers with PNALT have significantly higher cytotoxic activity compared to patients with CHC with elevated ALT or healthy individuals. Since NKG2D expression by NK cells in PNALT patients was at normal level we hypothesize that further factors may be involved in the regulation of their activity.

The pathogenesis of impaired CD8 response in CHC infection still remains partially understood. To further clarify this issue, in this study, we looked for activating and inhibitory receptor expression by CD8+ T cells. Our results show that CD8 T cell triggering can be hindered by engagement of inhibitory natural killer cell receptors, which are expressed on previously activated CD8 T cells. Although we found no differences in the expression of inhibitory receptor expression by CD8+ T cells between healthy individuals and HCV infected patients, but we found that activating NK receptors are expressed at significantly lower frequencies on CD8+ T cells during CHC infection. These findings suggest that decreased expression of activating NK receptors may play a role in HCV infection, possibly by inhibiting CD8 T-cell triggering.

Other factors such as cytokines may also play an important role during CHC infection[30-32]. IL-10 has largely been appreciated for its direct and indirect inhibitory effects on several T cell responses. IL-10 has been shown to contribute to regulation of immunopathology. The increased production of IL-10 by CD8+ T cells in CHC patients with persistently normal ALT reported in this paper is supposed to be important for limiting CD8 T cell response and contributing to asymptomatic virus carrier state. The NK cells of CHC carriers with PNALT produced significantly higher level of IL-4 and TNF-α, together with high cytotoxic activity compared to patients with CHC infection. We showed that cytokine pattern of NK cells and CD8+ T cells are

shifted towards a virus-permissive profile in patients with CHC infection which may ultimately contribute to HCV chronicity.

A major unresolved issue is the exact role of immune cells in different patient populations with acute and chronic hepatitis C virus infection. Further investigations are needed to clear whether NK, NKT-like and CD8+ T cells expressing various activating and inhibitory receptors could lead to viral clearance.

In conclusion we found complex dysregulation of activating and inhibitory receptor expression, such as decreased NKG2D and CD160 activating receptor expression and increased KIR2DL3 inhibitory receptor expression by NK and cytotoxic T cells in patients with chronic hepatitis C contributing to defective cellular immune functions. NKG2D receptor expression was significantly elevated in CHC infected patients with persistently normal ALT suggesting an important pathway for sustaining NK and CD8 T cell function and its critical role in protection against disease progression.

COMMENTS

Background

The host immune response to hepatitis C virus (HCV) involves both innate and adaptive arms of the immune system. Natural killer (NK) cells are key components of the innate antiviral immune response.

Research frontiers

To better characterize the immune defects underlying chronic viral persistence, the authors focus their analysis on killer inhibitory and activating receptor expression in patients with chronic HCV (CHC) infection with elevated alanine aminotransferase (ALT) and also in patients with CHC carriers with persistently normal ALT.

Innovations and breakthroughs

The authors found complex dysregulation of activating and inhibitory receptor expression by NK and cytotoxic T cells in patients with chronic hepatitis C contributing to defective cellular immune functions.

Applications

The percentage of Treg cells, KIR2DL3, ILT-2, KIR3DL1, CD160, NKG2D, NKG2C expressing NK, T and NKT-like cells, cytokine production and NK cytotoxicity were determined by flow cytometry.

Terminology

Persistently normal ALT was defined as ALT < 30 IU/L in men, ALT < 19 IU/L in women measured every 3 mo over a 18-mo period.

Peer-review

The manuscript described some phenotypical and functional differences in peripheral lymphocyte subsets between CHC patients with high and normal ALT. They describe a different expression in one NK activating receptor that could be related with TGF-β1 regulation. The manuscript is well written and the methodology is properly done.

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ORIGINAL ARTICLE

Basic Study

Gastric emptying, postprandial blood pressure, glycaemia and splanchnic flow in Parkinson's disease

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Author contributions: Trahair LG, Kimber TE, Horowitz M and Jones KL were involved in the conception and design of the study, and drafted of the manuscript; Trahair LG and Kimber TE were also involved in coordination and subject recruitment; Trahair LG and Flabouris K collected data; Trahair LG, Horowitz M and Jones KL performed statistical analysis; all authors interpreted the data; Jones KL had overall responsibility for the study.

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Abstract

AIM: To determine gastric emptying, blood pressure, mesenteric artery blood flow, and blood glucose responses to oral glucose in Parkinson's disease.

METHODS: Twenty-one subjects (13 M, 8 F; age 64.2 ± 1.6 years) with mild to moderate Parkinson's disease (Hoehn and Yahr score 1.4 ± 0.1, duration of known disease 6.3 ± 0.9 years) consumed a 75 g glucose drink, labelled with 20 MBq ^{99m}Tc-calcium phytate. Gastric emptying was quantified with scintigraphy, blood pressure and heart rate with an automated device, superior mesenteric artery blood flow by Doppler ultrasonography and blood glucose by

glucometer for 180 min. Autonomic nerve function was evaluated with cardiovascular reflex tests and upper gastrointestinal symptoms by questionnaire.

RESULTS: The mean gastric half-emptying time was 106 ± 9.1 min, gastric emptying was abnormally delayed in 3 subjects (14%). Systolic and diastolic blood pressure fell (P < 0.001) and mesenteric blood flow and blood glucose (P < 0.001 for both) increased, following the drink. Three subjects (14%) had definite autonomic neuropathy and 8 (38%) had postprandial hypotension. There were no significant relationships between changes in blood pressure, heart rate or mesenteric artery blood flow with gastric emptying. Gastric emptying was related to the score for autonomic nerve function (R = 0.55, P < 0.01). There was an inverse relationship between the blood glucose at t = 30 min (R = -0.52, P < 0.05), while the blood glucose at t = 180 min was related directly (R = 0.49, P < 0.05), with gastric emptying.

CONCLUSION: In mild to moderate Parkinson's disease, gastric emptying is related to autonomic dysfunction and a determinant of the glycaemic response to oral glucose.

Key words: Gastric emptying; Hypotension; Parkinson's disease; Blood pressure; Glucose

Core tip: We measured gastric emptying, blood pressure and blood glucose responses to a glucose drink in 21 patients with mild-to-moderate Parkinson's disease. Gastric emptying was shown to be abnormally delayed 3 patients and 40% had postprandial hypotension - a fall in systolic blood pressure > 20 mmHg after the glucose drink. We demonstrated relationships between gastric emptying and autonomic dysfunction, so that slower gastric emptying was associated with greater autonomic dysfunction, as well as relationships between the blood glucose response with gastric emptying.

Trahair LG, Kimber TE, Flabouris K, Horowitz M, Jones KL. Gastric emptying, postprandial blood pressure, glycaemia and splanchnic flow in Parkinson's disease. *World J Gastroenterol* 2016; 22(20): 4860-4867 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i20/4860.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i20.4860

INTRODUCTION

While gastrointestinal dysfunction occurs frequently in Parkinson's disease (PD)[1,2], the prevalence of abnormally delayed gastric emptying (GE) remains uncertain because of substantial variations in both the cohorts studied and the methodology used to quantify GE. Delayed GE has been associated with upper gastrointestinal and motor symptoms, as well as impaired absorption of dopaminergic therapy[3,4].

There is little or no information about the potential impact of GE in two other areas: postprandial blood pressure (BP) and glycaemia. Postprandial hypotension (PPH), a fall in systolic BP of ⩾ 20 mmHg within 2 h of a meal[5], was reported for the first time in 1977 in a patient with PD[6] and is a clinically important disorder, predisposing to syncope and falls and being associated with increased mortality[7]. PPH may occur frequently in PD, but information is limited[8]. It has also been suggested that PPH represents an "early" marker of autonomic dysfunction in PD[8,9]. Our studies have established that GE is pivotal to the regulation of postprandial BP- in healthy older subjects and patients with type 2 diabetes, the magnitude of the hypotensive response is greater when GE is relatively faster[10]. When glucose is infused intraduodenally in healthy older subjects at 1, 2 or 3 kcal/min, there is a substantial fall in systolic BP in response to the 2 and 3 kcal/min, but not the 1 kcal/min, load[11]. In contrast to the effect of GE, gastric distension attenuates the fall in BP[12], and consumption of water has been advocated as a treatment for PPH[7]. Only one study has evaluated the impact of GE on BP in PD and found no relationship in a cohort of 12 patients with mild to moderate disease[13]; BP was not a primary outcome in this study. The hypotensive response to a meal may relate to splanchnic blood pooling, as assessed by measurement of superior mesenteric artery (SMA) blood flow using Doppler ultrasound[14].

GE is an important determinant of postprandial glycaemia, which is a major contributor to "overall" glycaemic control in diabetes, as assessed by glycated hemoglobin[15]. Accordingly, in health[16], subjects with impaired glucose tolerance[16,17] and type 2 diabetes[17], when GE is faster, there is a greater initial glycaemic response. While diabetes per se does not appear to increase the propensity to PD[18], type 2 diabetes may be associated with greater impairments in postural stability and gait[19]. PD is associated with impaired insulin signalling in the brain[20] and drugs developed for the management of diabetes, particularly glucagon-like peptide-1 agonists, may have efficacy in treatment[21]. There is no information about the impact of GE on postprandial glycaemia in PD.

The primary aims of this study were to quantify the GE, BP, SMA flow and blood glucose responses to oral glucose in mild to moderate PD and evaluate the relationships of changes in BP and glycaemia with the rate of GE. We hypothesised that there would be a high prevalence of delayed GE, that consumption of glucose would result in a fall in BP and rises in both SMA flow and blood glucose, and that these responses would be related to GE.

Table 1 List of anti-Parkinsonian medications in 21 patients with Parkinson's disease *n* (%)

Drug	Patients
Pramipexole	11 (52)
Levodopa	9 (43)
Levodopa and Carbidopa	8 (38)
Levodopa, Carbidopa and Entacapone	4 (19)
Rasagiline	3 (14)
Amantadine	1 (5)
Apomorphone	1 (5)
Pregabalin	1 (5)
Selegiline	1 (5)

MATERIALS AND METHODS

Subjects

Twenty one subjects with mild to moderate PD were recruited through advertisements placed in a local Parkinson's newsletter, and outpatient referral by a neurologist (TK). Mild to moderate PD was defined as a score ⩽ 2.5 on the modified Hoehn and Yahr scale[22]. Subjects who were unable to move independently, or who had a history of falls, gastrointestinal disease (unrelated to Parkinson's), diabetes, significant respiratory or cardiac disease, alcohol abuse or epilepsy, were excluded. 13 males and 8 females, age 64.2 ± 1.6 years (range: 51-77 years), body mass index (BMI) 25.2 ± 0.8 kg/m^2 (range: 20.3-34.5 kg/m^2) and known duration of PD 6.3 ± 0.9 years (range: 1-16 years), were studied. Two patients were receiving antihypertensive drugs, which were withdrawn for 24 h before the study day. Details of anti-Parkinsonian medication are summarised in Table 1. Four subjects had received deep brain stimulation for the management of their PD.

Protocol

At an initial screening visit 6-65 d before the study day, a medical history and staging of Parkinson's symptoms on the modified Hoehn and Yahr scale were performed by a neurologist (TK)[22] and a questionnaire to asses symptoms referrable to delayed GE completed[23,24].

On the study day, subjects attended the Department of Nuclear Medicine, Positron Emission Tomography and Bone Densitometry at the Royal Adelaide Hospital at 0830h after an overnight fast from solids (14 h) and liquids (12 h). Where possible, subjects were asked to withhold the morning dose of anti-Parkinsonian medication. On arrival, the subject was seated in front of a gamma camera and an IV cannula inserted into the left antecubital vein for blood sampling. An automated cuff was placed around the upper right arm to measure BP and HR. The subject was then allowed to "rest" for approximately 15 min[11]. At *t* = -3 min, the subject consumed a drink comprising 75 g glucose and 5 g 3-O-Methyl-D-gluco-pyranose (3-OMG) (Carbosynth, Berkshire, United Kingdom) dissolved in water (total drink volume 300 mL), labelled with 20 MBq ^{99m}Tc-calcium phytate (Radpharm Scientific, Belconnen, ACT, Australia) within 3 min. GE, BP, SMA blood flow and blood glucose were measured for 180 min following the drink. At *t* = 180 min, the IV cannula was removed and the subject given a meal. Evaluation of autonomic function, using standardised cardiovascular reflex tests[25], was then performed, prior to the subject leaving the laboratory.

The protocol was approved by the Research Ethics Committee of the Royal Adelaide Hospital, and each subject provided written, informed consent prior to their inclusion. All experiments were carried out in accordance with the Declaration of Helsinki.

Measurements

Gastric emptying: Radioisotopic data was acquired for 180 min following consumption of the drink (60 s frames between *t* = 0-60 min, then 180 s frames from *t* = 60-180 min), where *t* = 0 was the time of completion of the drink. Data were corrected for subject movement, radionuclide decay and γ-ray attenuation[26]. A region-of-interest was drawn around the total stomach and gastric emptying curves (expressed as percentage retention over time) derived. The amount of the drink remaining in the total stomach at 15 min intervals between *t* = 0-180 min, as well as the 50% gastric emptying time (T_{50})[26], were calculated. The normal range for the T_{50} of this drink is 43-157 min, based on data in 21 healthy subjects (age 64.8 ± 1.8 years), matched for age (*i.e.,* within 2 years) to each subject with PD[17]. GE was considered to be abnormally fast or slow when the T_{50} was above, or below, this normal range.

Blood pressure and heart rate: BP and HR were measured using an automated BP monitor (DINAMAP ProCare 100, GE Medical Systems, Milwaukee, WI, United States), every 3 min during the "rest" period, and from *t* = 0-180 min. Baseline BP was calculated as an average of the three measurements obtained immediately prior to the consumption of the drink (*i.e.,* *t* = -9, *t* = 6 and *t* = -3 min)[11]. Maximum changes in BP and HR were calculated as the greatest change that occurred from baseline. Subjects were categorised according to the maximum fall in systolic BP following the drink, *i.e.,* those in which the fall was ⩽ 10 mmHg, > 10 mmHg but < 20 mmHg and ⩾ 20 mmHg. PPH was defined as a sustained (> 10 min) fall in systolic BP of ⩾ 20 mmHg[5].

Superior mesenteric artery blood flow: SMA flow was measured using a Logiq™ e ultrasound system (GE Healthcare Technologies, Sydney, NSW, Australia) and a 3.5C broad spectrum 2.5-4 MHz convex linear array transducer. Measurements were obtained immediately prior to the consumption of the drink (*t* = -3 min), every 15 min between *t* = 0-60 min, and then at *t* = 90 min, 120 min and 180 min. Blood flow (mL/min) was calculated automatically using the formula: π ×

$r^2 \times TAMV \times 60$, where R = the radius of the SMA and TAMV is the time-averaged mean velocity[14]. In all subjects two measurements were acquired by the same, experienced investigator (LT) at each time point.

Blood glucose: Venous blood was sampled immediately prior to the consumption of the drink (t = -3 min), every 15 min between t = 0-60 min and then at t = 90 min, 120 min and 180 min. Blood glucose (mmol/L) was determined immediately using a portable glucometer (Medisense Companion 2 m, Medisense Inc. Waltham, MA, United States). Results were classified, according to World Health Organisation criteria, as normal glucose tolerance (NGT) (fasting blood glucose < 6.1 mmol/L, and 2 h < 7.8 mmol/L), impaired fasting glucose (IFG) (fasting blood glucose < 7.0 mmol/L, but > 6.1 mmol/L), impaired glucose tolerance (IGT) (2 h blood glucose < 11.1 mmol/L, but > 7.8 mmol/L), or diabetes (fasting blood glucose ⩾ 7.0 mmol/L and/or 2 h blood glucose ⩾ 11.1 mmol/L)[27].

Upper gastrointestinal symptoms: Upper gastrointestinal symptoms assessed at the screening visit by questionnaire[23], included anorexia, nausea, early satiety, bloating, vomiting, abdominal pain, dysphagia, heart burn and acid regurgitation. Each was scored as: 0 = none, 1 = mild, 2 = moderate or 3 = severe, for a maximum score of 27[23].

Cardiovascular autonomic nerve function: Autonomic nerve function (ANF) was assessed using standardised cardiovascular reflex tests[25]. Parasympathetic function was evaluated by the variation (R-R interval) of the heart rate during deep breathing and the response to standing ("30:15" ratio). Sympathetic function was assessed by the fall in systolic BP in response to standing. Each of the results was scored according to age-adjusted predefined criteria as 0 = normal, 1 = borderline and 2 = abnormal for a total maximum score of 6. A score ⩾ 3 was considered to indicate definite autonomic dysfunction[25,28]. Orthostatic hypotension (OH) was defined as a sustained reduction in systolic BP of > 20 mmHg within 3 min of standing[29].

Statistical analysis: BP and HR were assessed as changes from baseline, whereas GE, SMA flow and blood glucose were analysed as absolute values. The maximum changes from baseline in BP, HR and blood glucose were also calculated. Areas under the curve (AUCs) were calculated for BP, HR, SMA flow and blood glucose using the trapezoidal rule. Changes in each variable over time were evaluated with ANOVA. Pearson's correlation was used to evaluate relationships between variables. Relationships of BP, upper gastrointestinal symptoms and glycaemia with GE were assessed using the GE T_{50}, given the observed overall linear pattern. A P value < 0.05 was considered significant in all analyses. The number of subjects included was based on power calculations derived from our previous study[10]. The statistical analysis was supervised and reviewed by a professional biostatistician. Data are presented as mean ± SE.

RESULTS

The studies were well tolerated and no adverse events were reported. The mean Hoehn and Yahr score was 1.4 ± 0.1 (range: 1-2.5) and duration of known PD 6.3 ± 0.9 years (range: 1-16 years). Three subjects were unwilling, or unable to withhold their morning anti-Parkinson medications because of the risk of significant motor dysfunction. Three subjects had definite autonomic neuropathy, in 10 subjects the score was ⩾ 2; the mean ANF score was 1.8 ± 0.3 (range: 0-5); 5 subjects had OH. Eight subjects had PPH. In another 8, the maximum fall was > 10 mmHg but < 20 mmHg and in 5 subjects the fall was < 10 mmHg. Four of the 5 subjects with OH also had PPH. The mean score for upper gastrointestinal symptoms was 1.5 ± 0.4 (range: 0-5).

Gastric emptying

Gastric emptying of the drink approximated an overall linear pattern. The T_{50} was 106 ± 9.1 min. In three subjects, GE (T_{50}) was abnormally slow; no subject had abnormally rapid GE.

Blood pressure and heart rate

Baseline systolic BP was 116.9 ± 2.4 mmHg. Following the drink, there was a transient modest rise, followed by a fall, in systolic BP (P < 0.001, Figure 1A), which was sustained until the end of the study. The maximum fall was -18.6 ± 2.0 mmHg, occurring at t = 76.5 ± 12.8 min.

Baseline diastolic BP was 69.1 ± 1.6 mmHg. Following the drink, there was a transient initial rise, and then a fall, in diastolic BP (P < 0.001, Figure 1B), with a nadir between t = 30-45 min, which was sustained until the end of the study. The maximum fall in diastolic BP was -15.6 ± 0.9 mmHg, occurring at t = 85.9 ± 11.6 min.

Baseline heart rate was 69.5 ± 2.1 BPM. Following the drink, there was an increase in heart rate (P < 0.001, Figure 1C), which had returned to baseline by approximately t = 60 min. The maximum increase in HR was 9.5 ± 0.7 BPM occurring at 75.6 ± 13.3 min.

Superior mesenteric artery blood flow

Baseline SMA flow was 565.0 ± 62.5 mL/min. Following the drink, there was a prompt increase in SMA flow (P < 0.001, Figure 2), which had returned to baseline by t = 180 min. The maximum SMA flow was 1208.8 ± 123.0 mL/min, occurring at t = 54.8 ± 8.1 min.

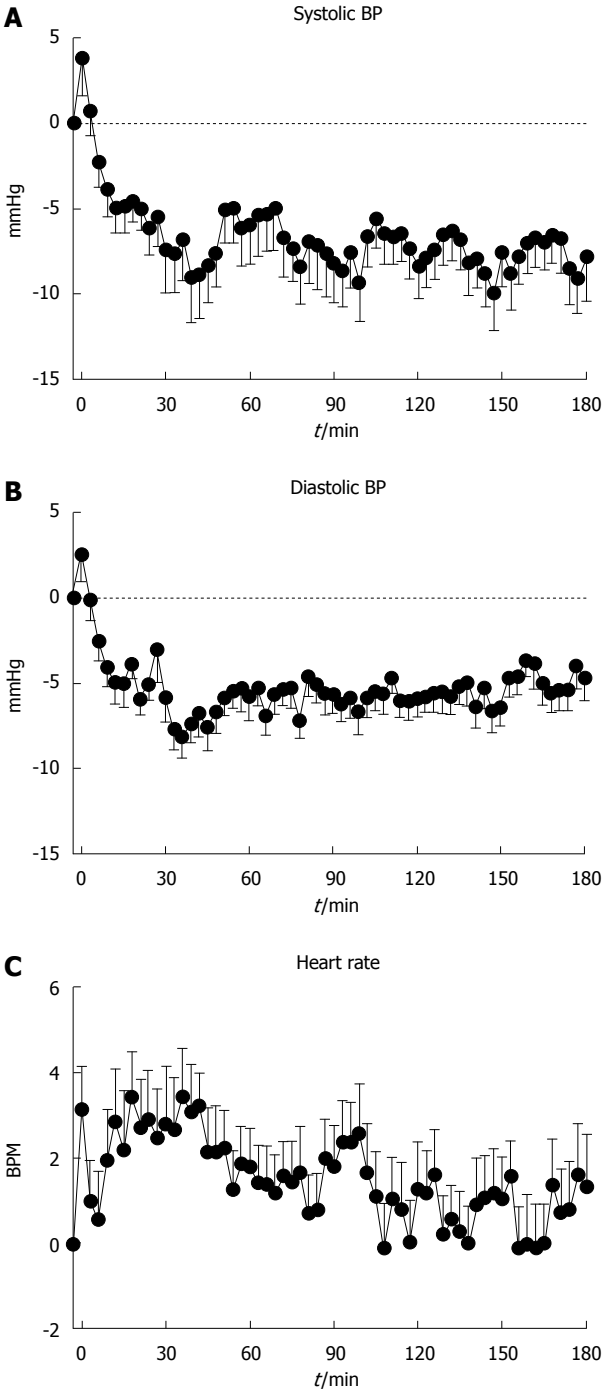

Figure 1 Systolic blood pressure (A), diastolic blood pressure (B) and heart rate (C) immediately before and after 75 g oral glucose load in 21 patients with Parkinson's disease. BP: Blood pressure.

Blood glucose

Baseline blood glucose was 5.6 ± 0.1 mmol/L. Following the drink, there was an increase in blood glucose (P < 0.001, Figure 3), which had returned to baseline by t = 180 min. The maximum blood glucose was 10.2 ± 0.5 mmol/L, occurring at 48.9 ± 4.0 min. Five subjects had IGT, 2 had both IFG and IGT and 1 had "marginal" diabetes (fasting and 2 h blood glucose of 7.1 mmol/L and 11.1 mmol/L, respectively).

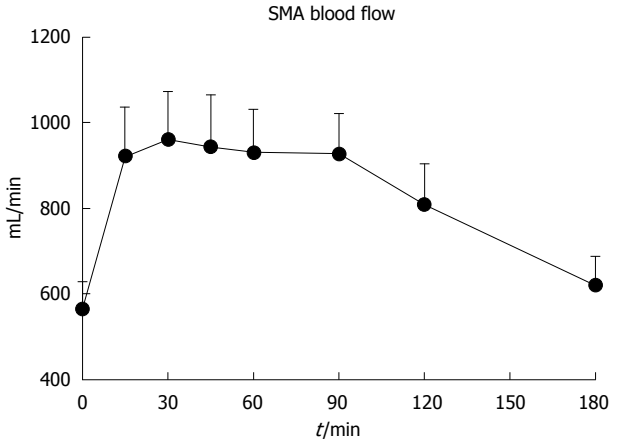

Figure 2 Superior mesenteric artery blood flow immediately before and after 75 g oral glucose load in 21 patients with Parkinson's disease. SMA: Superior mesenteric artery.

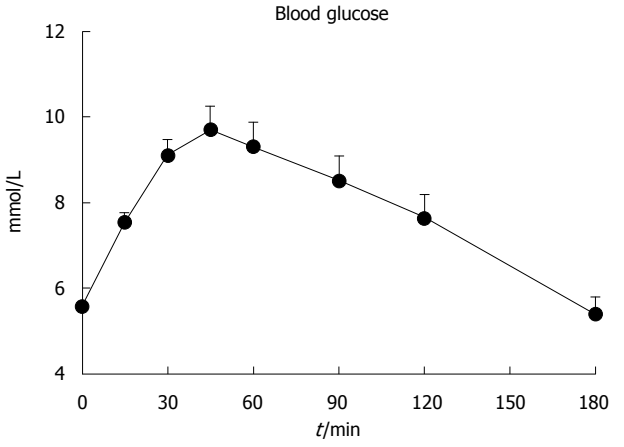

Figure 3 Blood glucose immediately before and after 75 g oral glucose load in 21 patients with Parkinson's disease.

Relationships between variables

There were no significant relationships between the changes in systolic BP, diastolic BP, HR or SMA flow at any time point (absolute values and AUCs). The T_{50} was related directly to the ANF score (R = 0.55, P < 0.01, Figure 4). Upper gastrointestinal symptoms were also related to the score for ANF (R = 0.45, P < 0.05), but not GE.

There was an inverse relationship between the blood glucose at t = 30 min (R = -0.52, P < 0.05, Figure 5), while the blood glucose at t = 180 min (but not 120 min) was related directly (R = 0.49, P < 0.05) to the T_{50}.

There were no significant relationships between T_{50}, Hoehn and Yahr score, duration of disease, or age.

DISCUSSION

Our study has quantified the GE, BP, SMA and glycaemic responses to oral glucose in mild to moderate

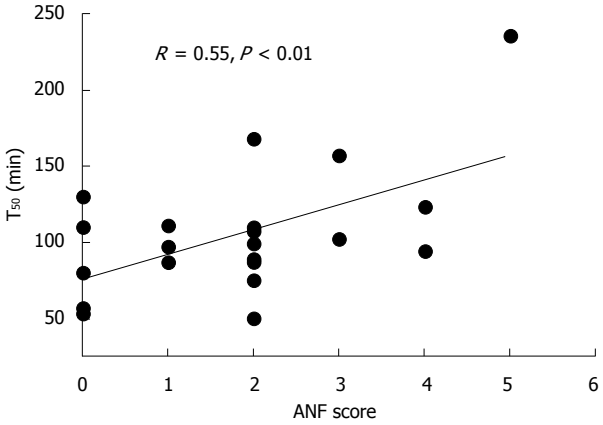

Figure 4 Relationship between gastric half emptying time (GE T_{50}) and autonomic nerve function score (R = 0.55, P < 0.01). ANF: Autonomic nerve function.

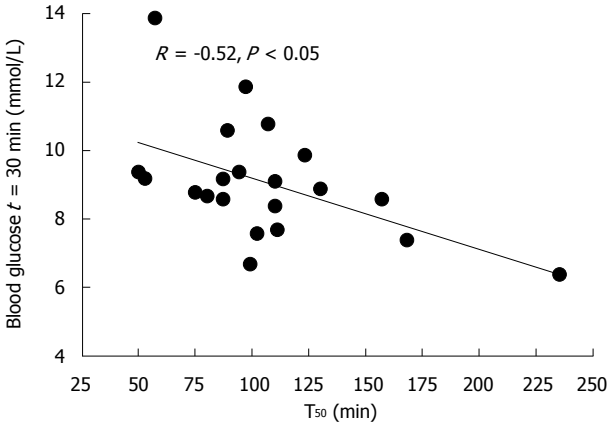

Figure 5 Relationship between the absolute blood glucose at t = 30 min with the gastric half emptying time (GE T_{50}) (R = -0.52, P < 0.05).

PD. In the majority of patients, oral glucose induced a significant fall in systolic BP; *i.e.,* in 16 of 21 patients (76%), this fall was > 10 mmHg and 8 (38%) had PPH. GE of glucose was abnormally delayed in 3 patients (14%), a prevalence lower than we anticipated and slower in those patients with cardiovascular autonomic neuropathy, and gastric emptying was not accelerated in any subject. There was, however, no relationship between the magnitude of the fall in BP with GE. A relationship between the initial glycaemic response to glucose with GE, comparable to that observed in subjects without PD, was demonstrated.

The outcome of studies relating to the prevalence of disordered GE in PD is inconsistent. We measured GE using the "gold-standard" technique of scintigraphy and, while a liquid, rather than a solid, "meal" was used, the precision of solid and high-nutrient liquid meals in the diagnosis of delayed GE appears comparable[30]. It should, however, be recognised that our definition of delayed GE - a T_{50} that was greater than the range observed in healthy subjects, was deliberately stringent, so that more modest gastric motor function cannot be excluded. The observations of a relationship between GE and the severity of autonomic dysfunction and the high prevalence of autonomic dysfunction are not surprising. The pathophysiology of disordered GE in PD is heterogeneous - alpha-synuclein aggregation, abnormalities in the dorsal motor nucleus of the vagus and enteric nervous system, and drugs such as L-dopa may all be important[2]. As with previous studies, there was no significant relationship between GE and the duration of PD[31]. Patients had mild upper gastrointestinal symptoms, possibly in part because the majority were studied off dopaminergic therapy, although symptoms were more common in patients with impaired ANF.

The high prevalence of PPH is comparable to that reported previously - 8 subjects had PPH and the fall in systolic BP was ⩾ 10 mmHg in 16 of 21 (76%) subjects[8]. That the latter may have adverse consequences, even in apparently "asymptomatic" patients[32], dictates the need for greater recognition. We did not observe a relationship between the magnitude of the fall in BP and GE, for which there are a number of potential explanations. Baseline systolic BP was in most cases "normal", which is predictive of a smaller postprandial fall[33]. We have demonstrated in healthy older subjects that the relationship between the fall in BP and the rate of duodenal glucose delivery is non-linear, so that a "threshold" between 1-2 kcal/min must be exceeded to elicit a hypotensive response[11]. In the current study, based on the T_{50}, GE was ⩾ 2 kcal/min in only 4 subjects. Hence, it would be appropriate to re-evaluate this hypothesis further in a larger group of patients. The current study certainly does not exclude the possibility that PD patients with relatively more rapid GE are at increased risk for PPH.

There was an approximate doubling in SMA flow following the glucose drink, as anticipated. In healthy subjects and patients with autonomic failure[34], comparable increases in SMA flow have been observed, but a reduction in BP was only evident in patients with autonomic failure, probably reflecting inadequate sympathetic compensation[34]. The absence of a relationship between BP and SMA flow, may reflect the relatively narrow distribution of the rises in SMA flow, and modest size of the cohort. OH is a frequent manifestation of autonomic involvement in PD and a concordance of PPH and OH in PD has been reported[8], and supported by our study.

The relationship between the initial glycaemic response to the drink and the rate of GE in PD is consistent with observations in health[16], impaired glucose tolerance[16,17], and type 2 diabetes[17] as well as the effect of delayed GE on the absorption of L-dopa in PD[2]. It is now recognised that postprandial glycaemic excursions are a major determinant of overall glycaemic control in type 2 diabetes, assuming increasing importance as glycated hemoglobin normalises[15]. Eight of our 21 subjects (38%) had either impaired glucose tolerance (7 subjects) or

"marginal" diabetes (1 subject); that the blood glucose level at 180 min, but not 120 min, was inversely, rather than directly, related to GE, presumably reflects higher insulin levels achieved earlier, associated with insulin resistance[16]. In healthy subjects an inverse relationship is evidence at 120 min after a 75 g oral glucose load[17]. The recognition that GE is a determinant of glycaemia in PD is not surprising, but potentially important- slower GE, including that induced by dopaminergic therapy, would potentially be advantageous in optimising glycaemic control in type 2 patients with PD. Interestingly, GLP-1 agonists, such as exenatide BD which are undergoing evaluation of their efficacy in the management of PD[21], diminish postprandial glycaemic excursions primarily by slowing GE[35].

In interpreting our observations, it should be recognised that 3 subjects did not withdraw their medication, which may represent a cofounder. We also did not include a control (water) drink because of potential ethical concerns. A normal range for GE allowed the prevalence of disordered GE in PD to be determined - a formal control group was not included because the focus of the present study was on relationships between variables within the Parkinson's group. As discussed, one subject had diabetes, based on fasting and 2 h blood glucose, but these levels were only marginally above the diagnostic cut-offs and this subject was not excluded.

In conclusion, in this unselected population of patients with mild to moderate PD, GE was delayed in only a minority, oral glucose induced a substantial reduction in BP, as well as rises in SMA flow and blood glucose, and GE was an important determinant of the glycaemic, but not the BP, response.

COMMENTS

Background

Delayed gastric emptying is recognised as a sequela of Parkinson's disease (PD), but its prevalence remains uncertain and the potential impact on both postprandial blood pressure and glycaemia have not been evaluated. Postprandial hypotension is known to occur frequently in PD and may be influenced by changes in superior mesenteric artery blood flow.

Research frontiers

Delayed gastric emptying in PD is associated with fluctuations in motor response, upper gastrointestinal symptoms and impaired absorption of dopaminergic therapy. The pathophysiology of postprandial hypotension is poorly defined, but the magnitude of the fall in blood pressure is known to be dependent on the rate of gastric emptying as well as changes in superior mesenteric artery blood flow.

Innovations and breakthroughs

The outcome of studies investigating the prevalence of disordered gastric emptying in PD have been inconsistent, at least in part reflecting variations in the cohorts studied and methodology employed to measure gastric emptying. The authors have measured gastric emptying using the "gold-standard" technique of scintigraphy in a well defined cohort of patients with PD to address these limitations. Furthermore, no study in PD has assessed changes in blood pressure and superior mesenteric artery blood flow following oral glucose.

Applications

The recognition that gastric emptying is a determinant of glycaemia is of importance to the management of glycaemic control in patients with PD who have type 2 diabetes. The authors identified a high prevalence of postprandial hypotension in this population, and given the substantial adverse sequale associated with this condition, this represents an important consideration in the management of PD with likely autonomic involvement.

Terminology

Postprandial hypotension, a fall in systolic blood pressure > 20 mmHg occurring within two hours of a "meal".

Peer-review

This is an excellent manuscript which comprehensively describes a very well-conducted investigation that is clinically relevant.

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ORIGINAL ARTICLE

Basic Study

Wortmannin influences hypoxia-inducible factor-1 alpha expression and glycolysis in esophageal carcinoma cells

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Abstract

AIM: To investigate the influence of phosphatidylinositol-3-kinase protein kinase B (PI3K/AKT)-HIF-1α signaling pathway on glycolysis in esophageal carcinoma cells under hypoxia.

METHODS: Esophageal carcinoma cell lines Eca109 and TE13 were cultured under hypoxia environment, and the protein, mRNA and activity levels of hypoxia inducible factor-1 alpha (HIF-1α), glucose transporter 1, hexokinase-Ⅱ, phosphofructokinase 2 and lactate dehydrogenase-A were determined. Supernatant lactic acid concentrations were also detected. The PI3K/AKT signaling pathway was then inhibited with wortmannin, and the effects of hypoxia on the expression or activities of HIF-1α, associated glycolytic enzymes and lactic acid concentrations were observed. Esophageal carcinoma cells were then transfected with interference plasmid with HIF-1α-targeting siRNA to assess impact of the high expression of HIF-1α on glycolysis.

RESULTS: HIF-1α is highly expressed in the esophageal carcinoma cell lines tested, and with decreasing levels of oxygen, the expression of HIF-1α and the associated glycolytic enzymes and the extracellular lactic acid concentration were enhanced in the esophageal carcinoma cell lines Eca109 and TE13. In both normoxia and hypoxic conditions, the level of glycolytic enzymes

and the secretion of lactic acid were both reduced by wortmannin. The expression and activities of glycolytic enzymes and the lactic acid concentration in cells were reduced by inhibiting HIF-1α, especially the decreasing level of glycolysis was significant under hypoxic conditions.

CONCLUSION: The PI3K/AKT pathway and HIF-1α are both involved in the process of glycolysis in esophageal cancer cells.

Key words: Hypoxia-inducible factor-1 alpha; Hypoxia; Glycolysis; Esophageal neoplasms; Cell metabolism

Core tip: Fluorescence analysis, spectrophotometry, real-time PCR, Western blot and siRNA interference technology were used to investigate the influence of phosphatidylinositol-3-kinase protein kinase B-HIF-1α signaling pathway on glycolysis under hypoxia in esophageal carcinoma cells. The results obtained provide experimental evidence for the mechanism of glycolysis enhanced by hypoxia.

Zeng L, Zhou HY, Tang NN, Zhang WF, He GJ, Hao B, Feng YD, Zhu H. Wortmannin influences hypoxia-inducible factor-1 alpha expression and glycolysis in esophageal carcinoma cells. *World J Gastroenterol* 2016; 22(20): 4868-4880 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i20/4868.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i20.4868

INTRODUCTION

Esophageal cancer, as one of the most common malignant tumors in China with a poor prognosis, is characterized by fast invasion and early metastasis with chemotherapy and radiotherapy tolerance. Previous studies have confirmed that tumor hypoxia is an important factor leading to radiotherapy and chemotherapy resistance and that it promotes tumor invasion and metastasis and affects cell energy metabolism[1,2]. Tumor cells always prefer aerobic glycolysis metabolism to obtain energy, and this preference was named Warburg effect[3]. It enhances the ability of tumor cells to metabolize glucose to provide energy sources for their rapid proliferation and growth as well as help with their hypoxia tolerance. Synergies between tumor tissue hypoxia and the Warburg effect may play an important role in the promotion of tumor metastasis and resistance to chemotherapy.

Hypoxia-inducible factor-1 alpha (HIF-1α) is known as a key regulatory factor of tissue adaptation under hypoxia. It is highly expressed in most tumors and metastases and is inseparable from the glycolytic pathway of tumor cells[4]. The PI3K/AKT signaling pathway widely exists in cells, and it is involved in cell growth, proliferation, differentiation and regulatory signal transduction pathways. The pathway is also one of the most closely regulated pathways in cancer cells influencing glucose metabolism, in addition to its participation in the regulation of HIF-1α expression[5-7]. Therefore, we hypothesized that the PI3K/AKT pathway and HIF-1α may play a key role in the synergistic effect of hypoxia and the Warburg effect.

MATERIALS AND METHODS

Materials

Human esophageal carcinoma cell lines TE13 and Eca109 were purchased from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). Interference plasmid with HIF-1α-targeting siRNA was manufactured by the Kejira Corporation (Shanghai, China). Competent *E. coli* TOP10 cells were bought from the Bordi Corporation (Nanjing, China). The antibodies for HIF-1α, AKT, glucose transporter-1 (GLUT-1), lactate dehydrogenase-A (LDHA) and the secondary antibodies were obtained from Santa Cruz Biotechnology. The antibodies for HK-Ⅱ and p-AKT were obtained from Cell Signaling Technology. The GAPDH antibody was purchased from Bioworld. The Takara reverse transcription kit, the SYBR Green quantitative PCR kit, TRIzol and all the primers were obtained from the Shanghai to Betting Biotechnology Co., Ltd. A hypoxic incubator was purchased from Sanyo.

Cell lines

Esophageal carcinoma cell lines TE13 and Eca109 (2×10^5 cells/well) maintained in DMEM with 10% fetal bovine serum were covered with serum-free medium when the cells grew to 60% confluency and starved for 24 h. Three groups of adherent cells in the logarithmic growth phase were placed into the hypoxia incubator (5% CO_2, 1% O_2 and 94% N_2), and the cells were incubated for 6 h, 12 h, 24 h and 48 h. A corresponding blank control was also set up.

Cell transfection and colony selection

Two pairs of HIF-1α-siRNA oligonucleotide fragments were designed and synthesized according to the human HIF-1α gene sequence (GenBank No. NM001530). The sequences were 5′-GATCCCGAGGAAGAACTATGAACATAATTCAAGAGATTATGTTCATAGTTCTTCCTCTTTTTGGAT-3′ (sense strand) and 5′-AGCTATCCAAAAAGAGGAAGAACTATGAACATAATCTCTTGAATTATGTTCATAGTTCTTCCTCGG-3′ (antisense strand) for sequence one, and 5′-GATCCCGACTGATGACCAGCAACTTGATTCAAGAGATCAAGTTGCTGGTCATCAGTCTTTTTGGAT-3′ (sense strand) and 5′-AGCTATCCAAAAAGACTGATGACCAGCAACTTGATCTCTTGAATCAAGTTGCTGGTCATCAGTCGG-3′ (antisense strand) for sequence two.

To construct a plasmid on the basis of the pGCsi vector manual, TOP10 cells were amplified and agarose gel electrophoresis was performed to acquire the plasmids, which were named pGCsi-HIF-1 and pGCsi-HIF-2. They were routinely used to transfect the cell lines Eca109 and TE13. The cell transfection efficiencies were determined based on the green fluorescence as detected by fluorescence microscopy, and finally, the pGCsi-HIF-1 plasmid was selected as the follow-up interference plasmid. The plasmid pGCsi-HIF-1 and its negative control plasmids were transfected. The cell clones were batched and picked after four weeks. The results of RT-PCR and Western blot were combined. The plasmid pGCsi-HIF-1 and corresponding negative control plasmids were named TE13/shRNA, TE13/Neo, Eca109/shRNA and Eca109/Neo.

Drug effectiveness

Wortmannin at an experimental concentration of 2 μmol/L was incubated with the cells in a hypoxia incubator (1% O_2) for 12 h and the control group was cultured for the same time under normoxia.

Western blot analysis

Western blot analysis was performed to detect the protein expression of HIF-1α and the associated glycolysis genes. The proteins were conventionally extracted, transferred to membranes and incubated. The corresponding primary antibody concentrations were as follows: HIF-1α (1:500), HK-Ⅱ (1:1000), GLUT-1 (1:200), LDHA (1:200) and β-actin (1:4000). The secondary antibodies conjugated with HRP were goat anti-mouse (1:4000), goat anti-rabbit (1:4000) and rabbit anti-goat (1:5000). The signal was developed using ECL chemiluminescence.

Quantitative real-time PCR

To extract and purify the total RNA, TRIzol-blue reagent was used to extract the cells pretreated in each group according to the instructions of the TRIzol Kit. Then, 1 μg of total RNA was reverse transcribed with the RevertAid™ First Strand cDNA Synthesis Kit. The mRNA levels were determined by qRT-PCR using the Bio-Rad MJ Mini Opticon.

Activities of enzymes

The cells (5×10^5 cells/well) were washed twice in PBS and 500 μL of PBA was added after trypsin digestion. Sonication and centrifugation (10000 r/min) for 10 min were used to acquire the supernatant, and then the activities of LDH and HK were detected by colorimetry according to the kit instructions.

Spectrophotometry

Spectrophotometry was performed to detect the concentration of lactic acid in the supernatant of the nutrient solution.

Statistical analysis

Statistical analyses were performed using SPSS 18.0 software. Gray value analysis was performed with Tanon Gis software, and the ratio of the target band to the internal reference band represented the protein expression levels of the target gene. The differences in the expression of HIF-1α and the associated glycolytic enzymes, the activities of HK and LDH and the lactic acid concentration among the multiple groups were analyzed using an *F* test, and indicators between the two groups were compared using Student's *t*-test or *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Influence of oxygen concentration on the protein levels of HIF-1α and the associated glycolytic enzymes in esophageal cancer cells

Under hypoxic condition (1% O_2), the protein expression of HIF-1α in esophageal carcinoma cell lines increased gradually with time. After 12 h of hypoxia, its expression peaked and then maintained a high level. Then, it began to decrease after 48 h. The protein expression of GLUT-1 and HK-Ⅱ also elevated gradually after incubation under hypoxic conditions and reached its peak at a time between 12 and 24 h, whereas the protein change of LDHA did not follow a similar trend (Figure 1).

Influence of oxygen concentration on the lactic acid concentration in the supernatant of esophageal carcinoma cells

The lactic acid concentration in the supernatant of the cells increased in an upward trend with lower oxygen concentrations. When the culture time for the esophageal carcinoma cell lines (TE13 and Eca109) under hypoxia was extended for 6-48 h, the lactic acid concentration peaked at 12 h and was then maintained at that level or decreased slightly (Figure 2).

Effect of wortmannin on the mRNA expression of glycolytic enzymes in esophageal carcinoma cells

mRNA expression: Compared with a normoxic environment, the mRNA expression of HIF-1α and HK-Ⅱ under hypoxic conditions increased significantly in the esophageal carcinoma cell lines Eca109 and TE13. In both normoxic and hypoxic conditions, the mRNA expression of the enzymes in the group pretreated with wortmannin decreased slightly compared with that in the group without pretreatment ($P < 0.05$), but the differences among each group for GLUT-1 and LDHA at the mRNA level were not significant ($P > 0.05$) (Figure 3).

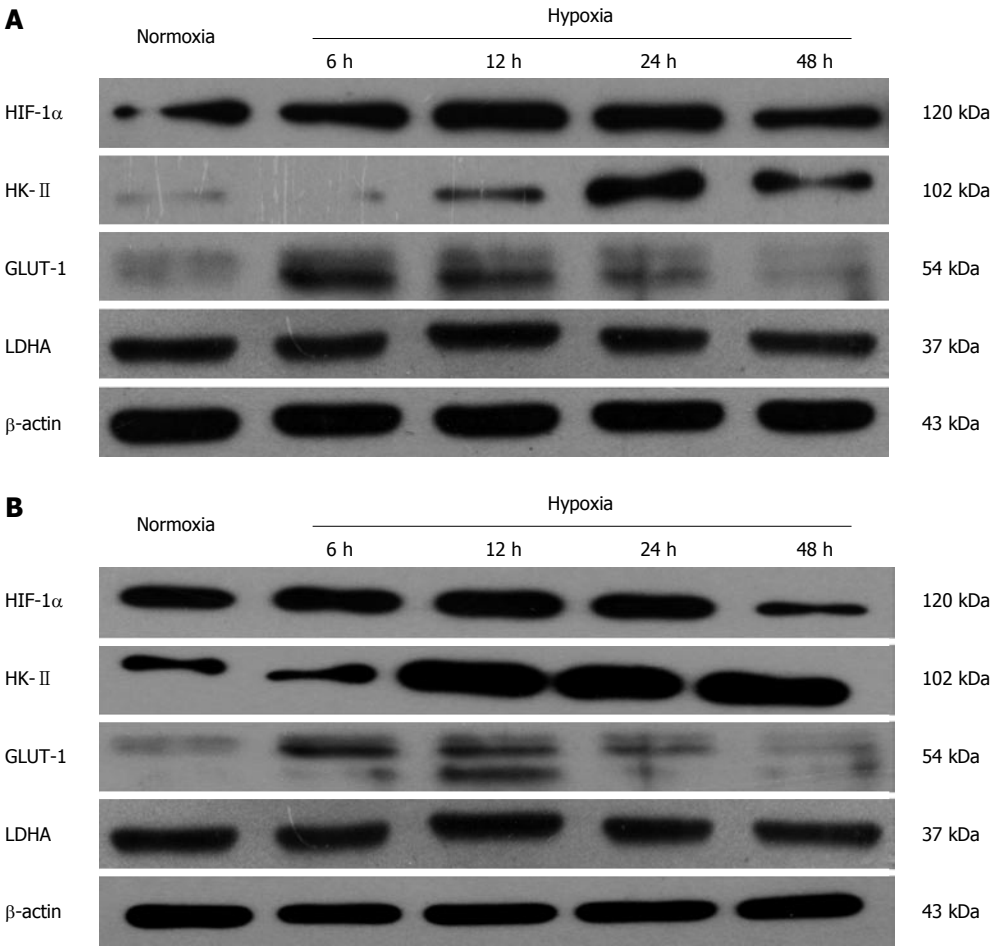


Figure 1 Expression of hypoxia inducible factor-1 alpha and glycolysis enzymes at different time points of hypoxia in Eca109 cells (A) or in TE13 cells (B). HIF-1α: Hypoxia inducible factor-1 alpha; HK-Ⅱ: Hexokinase Ⅱ; GLUT-1: Glucose transporter-1; LDHA: Lactate dehydrogenase-A.

Protein expression: Compared with groups not pretreated with wortmannin, the protein expression of HIF-1α in the pretreated esophageal carcinoma cell lines Eca109 and TE13 incubated under normoxic and hypoxic conditions was inhibited markedly ($P < 0.05$), and the protein expression of the hypoxic group was higher than that of the normoxic group ($P < 0.01$). Because the expression of HIF-1α was repressed most when a culture time of 12 h was used, we chose that time for the subsequent experiments.

The protein expression of HK-Ⅱ and GLUT-1 under normoxia or hypoxia in the groups pretreated with wortmannin was significantly reduced compared with those groups not pretreated ($P < 0.05$ between each of the groups). In the condition of hypoxia, the protein expression of LDHA among each group increased compared with the normal oxygen environment ($P < 0.05$). However, the difference between the group pretreated with wortmannin and the group not pretreated was not statistically significant ($P > 0.05$) (Figure 4).

Enzyme activities and lactic acid concentration

By detecting the activities of LDH and HK-Ⅱ in the esophageal carcinoma cell lines Eca109 and TE13 incubated for 12 h under normoxia and hypoxia, we found that the activities of LDH and HK in the groups pretreated with wortmannin all obviously declined compared with those in the group without pretreatment ($P < 0.05$) (Figure 5A). In the normoxic and hypoxic environments, the lactic acid concentrations in the pretreated group decreased markedly compared with those in the group without pretreatment ($P < 0.05$) (Figure 5B).

Impact on the mRNA expression of glycolytic enzymes after inhibiting the expression of HIF-1α in esophageal carcinoma cells

mRNA expression: Compared with the esophageal carcinoma cells of the untransfected group and the empty vector group, the mRNA expression of GLUT-1 and HK-Ⅱ in the Eca109/siRNA group obviously decreased ($P < 0.05$) (Figure 6). However, the decline in the mRNA expression of LDHA was too small to be significant. The expression of the associated genes in the untransfected group and the empty vector group under hypoxia were enhanced slightly, and the difference was considered statistically significant ($P < 0.05$).

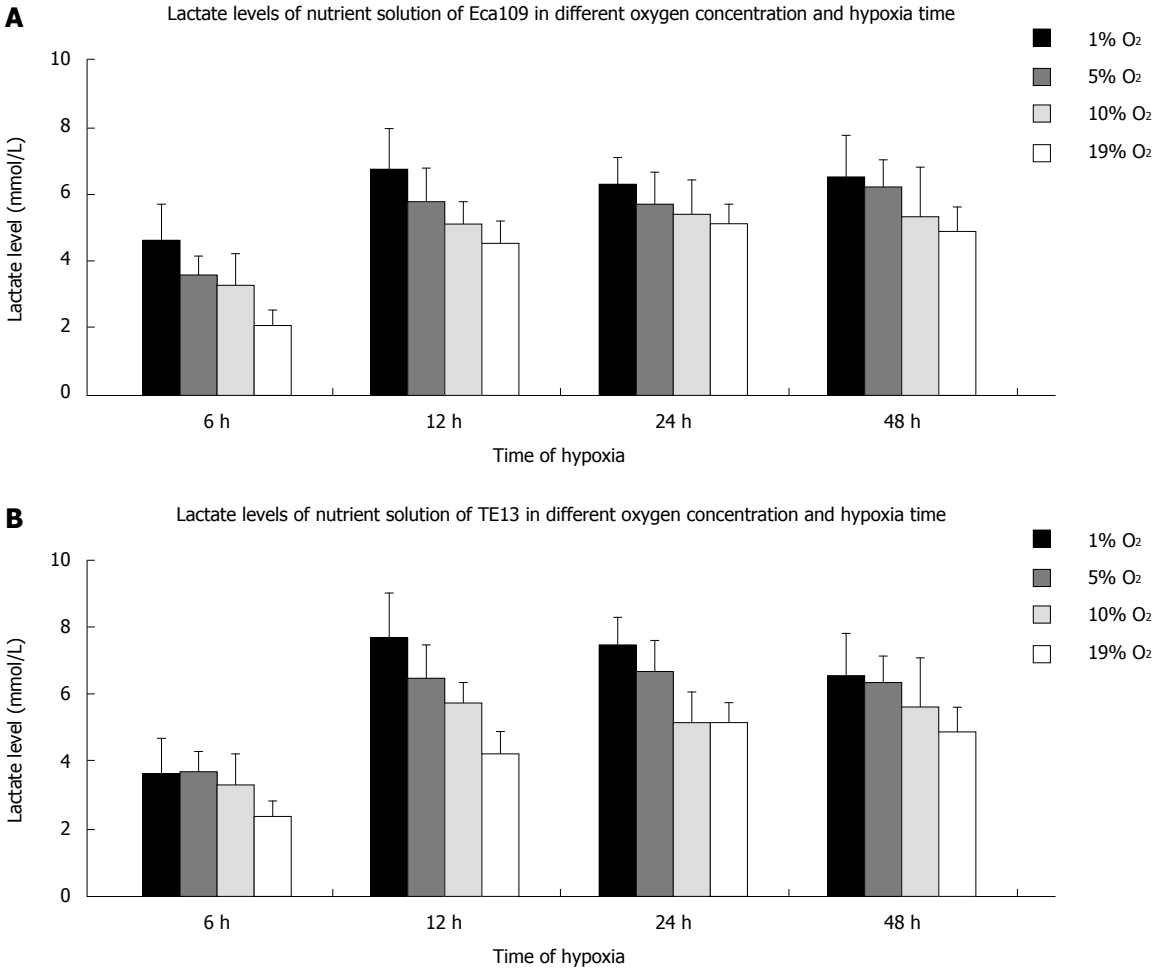


Figure 2 Lactic levels in culture medium of Eca109 cells (A) or TE13 cells (B) in different oxygen environments and at different time points as revealed by photocolorimetric method.

Protein expression: Compared with the TE13 and Eca109 groups, the protein expression of HIF-1α in the TE13/siRNA and Eca109/siRNA groups were clearly reduced, and hypoxia could not correct this phenomenon. Regardless of the oxygen concentration used for culturing, the expression of GLUT-1 and HK-Ⅱ at the protein level in the TE13/siRNA and Eca109/siRNA groups was obviously weaker than that in the control group ($P < 0.05$). However, the expression of LDHA in the group of TE13 and Eca109 under hypoxia was up-regulated significantly compared with that of the normal oxygen group ($P < 0.05$), but mildly declined after inhibiting HIF-1α compared to the cells that were not silenced. The results were not statistically significant ($P > 0.05$) (Figure 7).

Enzymatic activities and lactic acid concentration

The activities of LDH and HK-Ⅱ under normoxia or hypoxia in the Eca109/siRNA and TE13/siRNA groups obviously decreased compared with those of the untransfected and empty vector groups ($P < 0.05$), and the activities of the enzymes under hypoxia increased slightly in the untransfected and empty vector groups ($P > 0.05$) (Figure 8). Moreover, compared with the untransfected group in the normoxia and hypoxia environments, the lactic acid concentrations in the Eca109/siRNA and TE13/siRNA groups were clearly reduced ($P < 0.05$) (Table 1).

DISCUSSION

Hypoxia is one of the basic characteristics of solid tumor microenvironments. On one hand, hypoxia results from the increase in oxygen consumption caused by the rapid proliferation of malignant tumors. On the other hand, the abnormality of the vascular structure and function of tumors leads to a decrease in the blood and oxygen supply, which could also be an important factor further adding to the risk of hypoxia in tumors[8-10]. When tumor size is greater than 1 mm^3, there were a considerable number of tumor cells in a hypoxic state. Hypoxia in tumors is not only the consequence of pathophysiology but also an important initiating factor of malignant transformation and even metastasis in the development of tumors. Research has confirmed that hypoxia can promote oncogenes to produce functionally acquired mutations to cause high expression of the gene itself. It has also been found

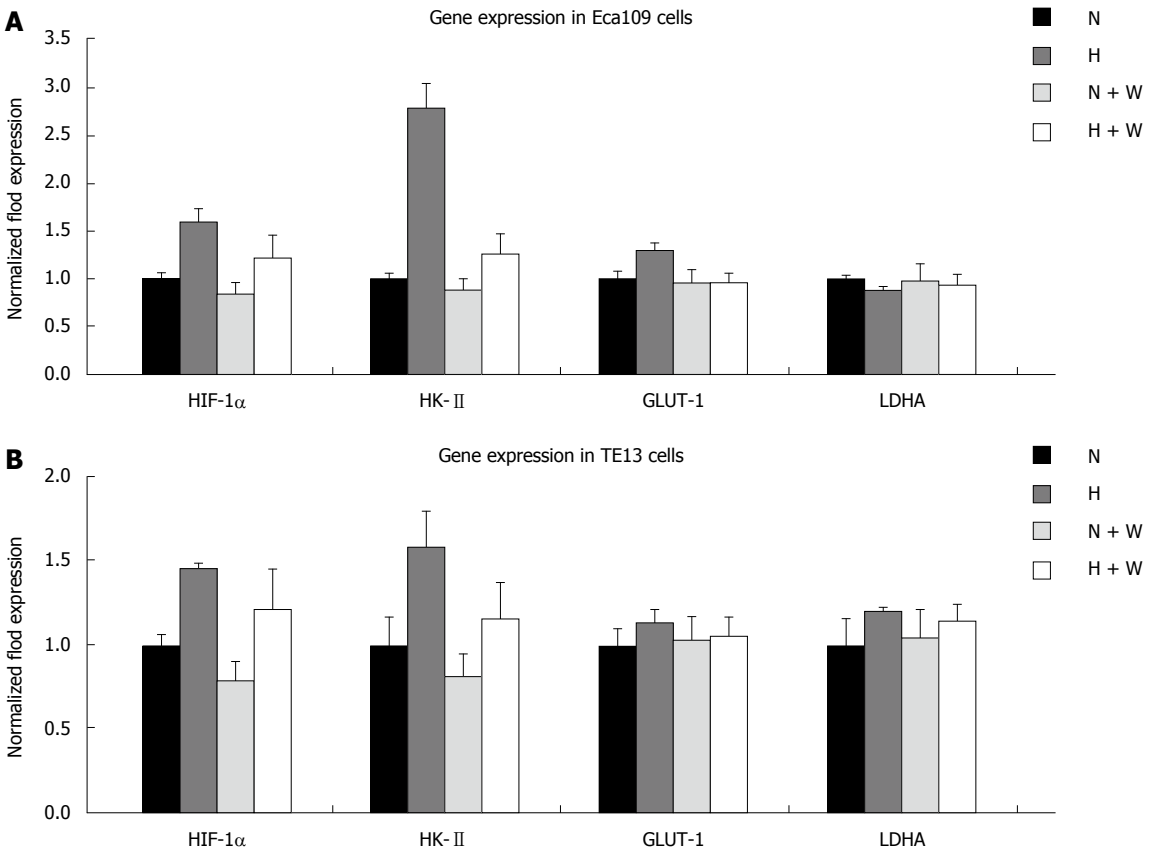
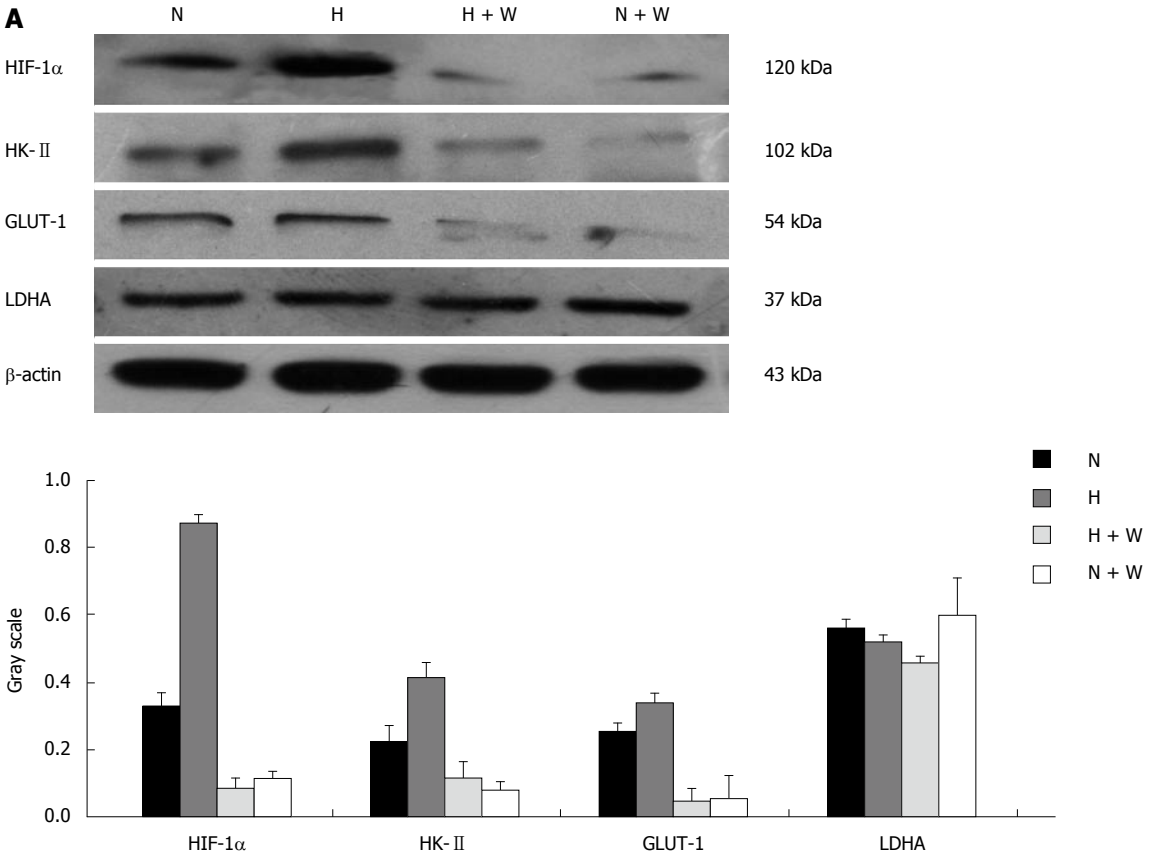


Figure 3 Quantitative real-time PCR analysis of the gene expression of hypoxia inducible factor-1 alpha and glycolysis (*n* = 3). HIF-1α: Hypoxia inducible factor-1 alpha; HK-Ⅱ: Hexokinase Ⅱ; GLUT-1: Glucose transporter-1; LDHA: Lactate dehydrogenase-A.



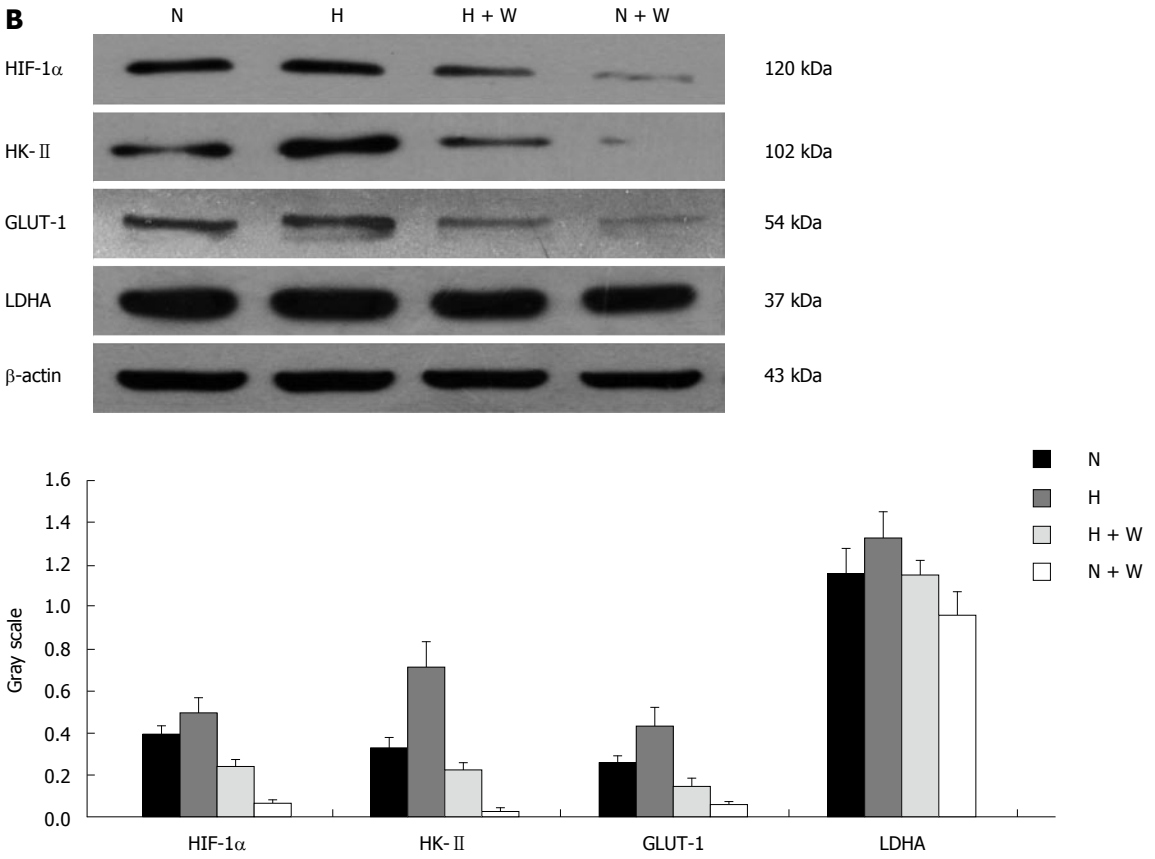
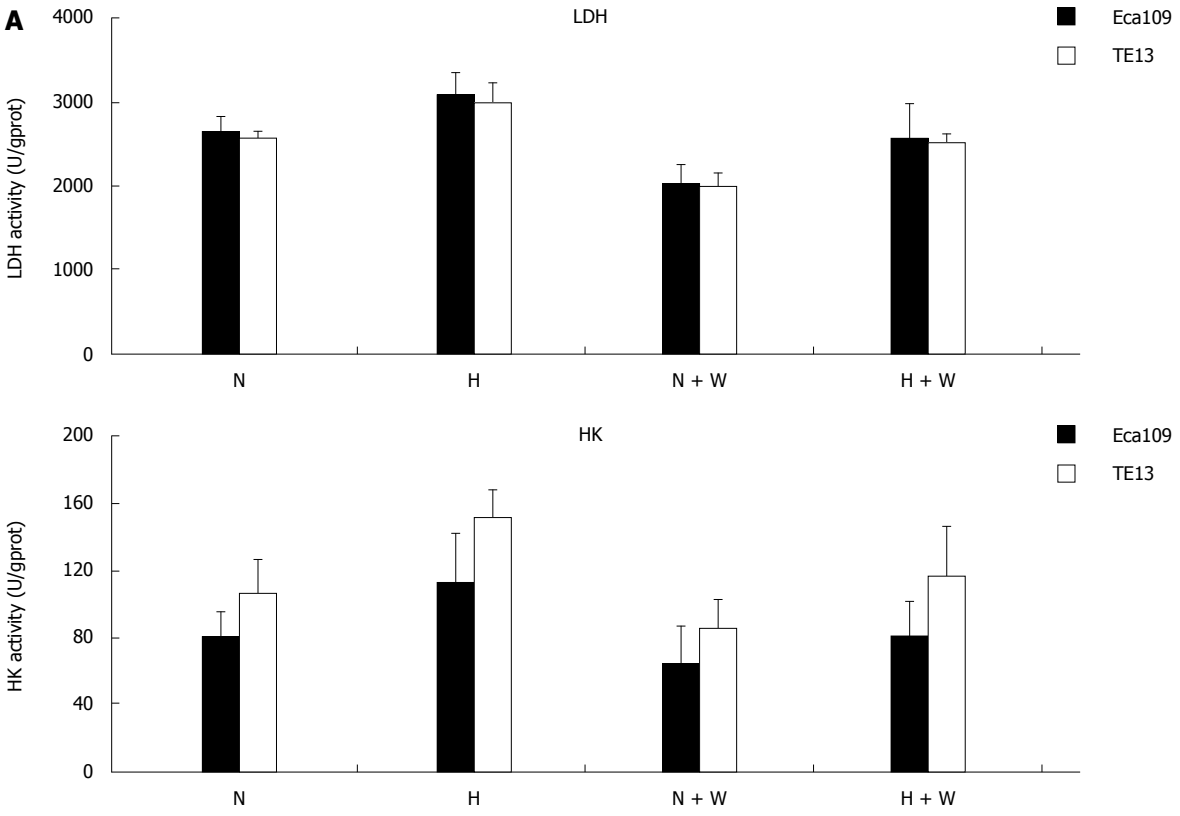


Figure 4 Effect of wortmannin on hypoxia inducible factor-1 alpha and glycolysis protein in Eca109 cells (*n* = 3) (A) and in TE13 cells (B) (*n* = 3). HIF-1α: Hypoxia inducible factor-1 alpha; HIF-1α: Hypoxia inducible factor-1 alpha; HK-Ⅱ: Hexokinase Ⅱ; GLUT-1: Glucose transporter-1; LDHA: Lactate dehydrogenase-A.



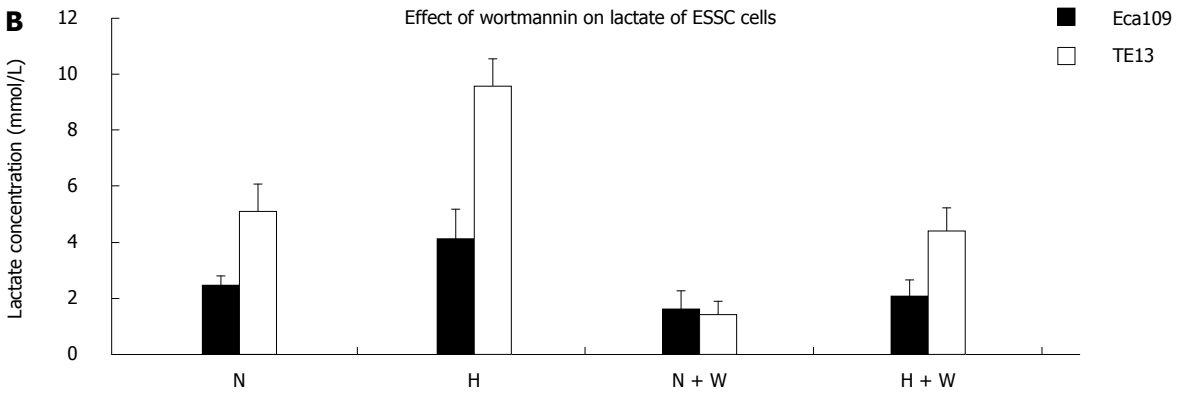


Figure 5 Spectrophotometry analysis of the activities of lactate dehydrogenase and hexokinase (*n* = 3) (A) or the supernatant lactic acid concentration in nutrient solution (*n* = 3) (B). HK: Hexokinase; LDH: Lactate dehydrogenase.

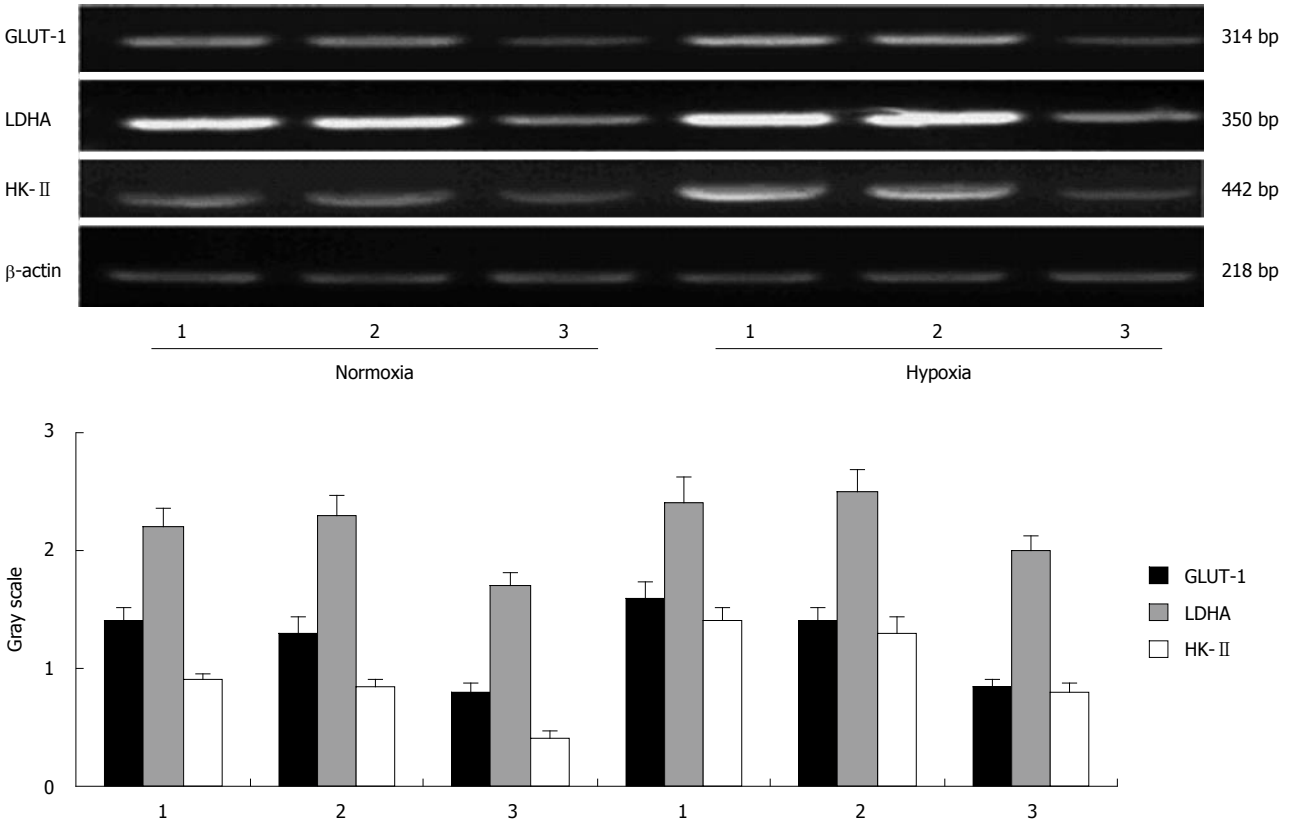


Figure 6 mRNA expression of glycolysis enzymes after hypoxia inducible factor-1 alpha inhibition (*n* = 3). 1: Eca109; 2: Eca109/neo; 3: Eca109/siRNA. HIF-1α: Hypoxia inducible factor-1 alpha; HK-Ⅱ: Hexokinase Ⅱ; GLUT-1: Glucose transporter-1; LDHA: Lactate dehydrogenase-A.

that the corresponding proteins, apart from the tumor suppressor genes, have functionally lacking mutations that down-regulate the expression of genes or weaken the activities of products. In addition, hypoxia can also promote the functionally lacking mutations of the gene associated with DNA repair to improve the mutation frequency of oncogenes and tumor suppressor genes; thus, it is involved in malignant transformations in tumor genesis[11,12]. Furthermore, hypoxia participates in the processes of invasion, metastasis, angiogenesis, immune evasion, and radiotherapy and chemotherapy resistance to affect prognosis[13,14]. Most normal tissues always use aerobic oxidation for energy metabolism in an anaerobic environment, and the glycolytic pathway started to mobilize only when the oxygen supply was insufficient. However, tumor cells prefer glycolysis to gain energy to meet their needs, regardless of the oxygen condition, which is known as the Warburg effect[3]. It is more convenient for tumor cells to gain energy for metabolism and survive under hypoxic conditions, which helps in the acquisition of hypoxia tolerance. The radiotherapy and chemotherapy resistance caused by low metabolic rates during hypoxia helps some tumor cells survive, which could be a source of tumor recurrence[15].

HIF-1 is a transcription factor that widely exists in

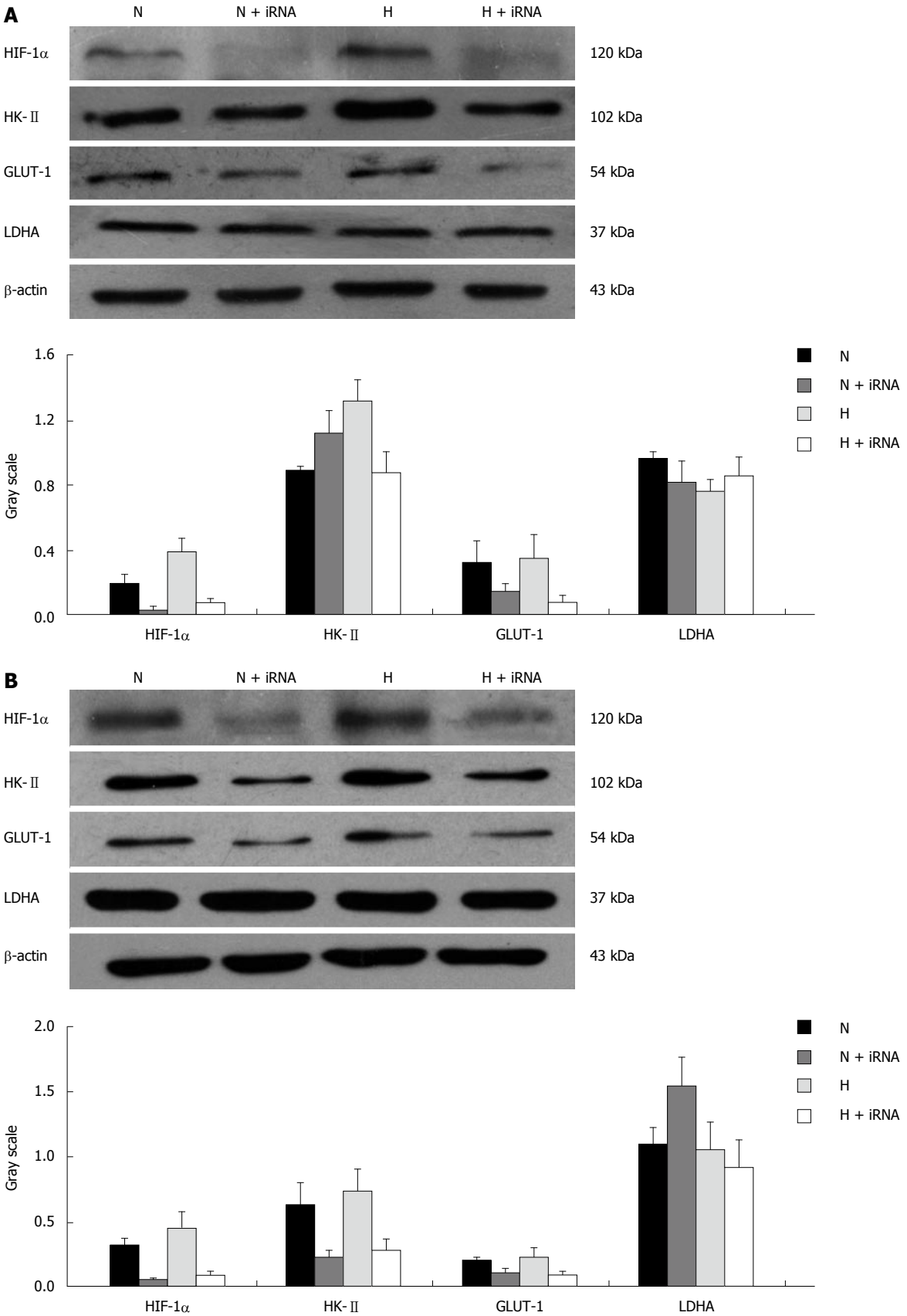


Figure 7 Protein expression of glycolysis enzymes. A: In Eca109 cells after HIF-1α inhibition (n = 3). N: Eca109 + 19% O_2; N + iRNA: Eca109/siRNA + 19% O_2; H: Eca109 + 1% O_2; H + iRNA: Eca109/siRNA + 1% O_2. B: In TE13 cells after HIF-1α inhibition (n = 3). N: TE13 + 19% O_2; N + iRNA: TE13/siRNA + 19% O_2; H: TE13 + 1% O_2; H + iRNA: TE13/siRNA + 1% O_2. HIF-1α: Hypoxia inducible factor-1 alpha; HK-Ⅱ: Hexokinase Ⅱ; GLUT-1: Glucose transporter-1; LDHA: Lactate dehydrogenase-A.

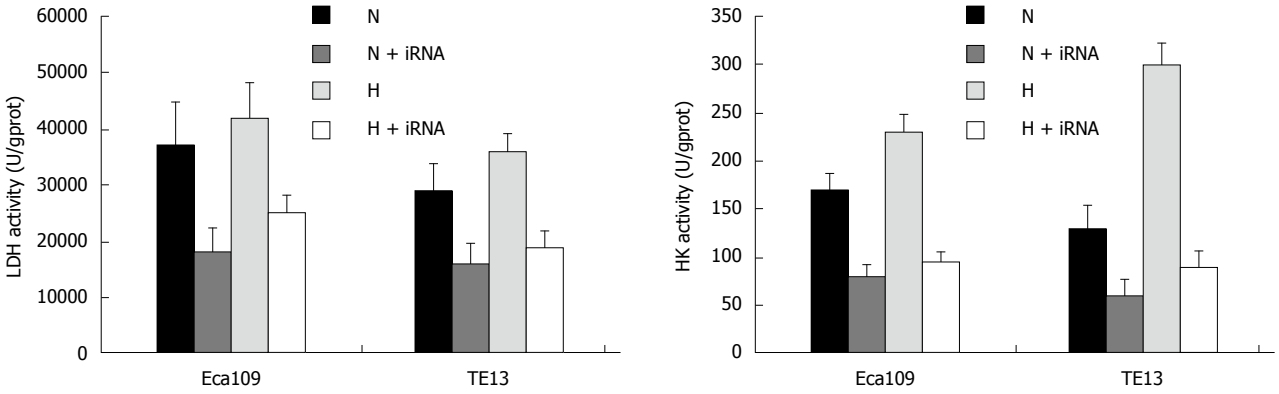


Figure 8 Western blot analysis of the activities of glycolysis enzymes after hypoxia inducible factor-1 alpha inhibition. HIF-1α: Hypoxia inducible factor-1 alpha; HK-Ⅱ: Hexokinase Ⅱ; GLUT-1: Glucose transporter-1; LDHA: Lactate dehydrogenase-A.

Table 1 The changes of supernatant lactic acid concentrations after restraining the expression of hypoxia inducible factor-1 alpha (n = 3)

	TE13		Eca109	
	TE13	TE13/siRNA	Eca109	Eca109/siRNA
A (19% O_2)	3.333 ± 0.833	1.933 ± 0.569[c]	4.867 ± 0.551	1.367 ± 0.346[c]
B (1% O_2)	6.433 ± 1.059[a]	1.567 ± 0.416	8.067 ± 1.160[a]	1.767 ± 1.242[a]

[a]$P < 0.05$, *vs* group A; [c]$P < 0.05$, *vs* non-interference group.

mammals and humans under hypoxic conditions and is a key player in the body's ability to adapt to hypoxia by promoting the expression of hypoxia-induced genes to elicit the specific response to hypoxia of tissues. HIF-1 consisted of HIF-1α and HIF-1β subunits. HIF-1α is not only peculiar to HIF-1 but also the oxygen regulator unit. The hypoxia-inducible genes regulated by HIF are involved in angiogenesis, erythropoiesis, energy metabolism, apoptosis, proliferation and other cellular processes. Previous studies have found that the related glycolytic genes regulated by HIF-1 include the GLUT-1, lactate dehydrogenase A (LDHA), phosphofructokinase 2 (PFK2), aldolase A, enolase 1, phosphoglycerate kinase 1 and glyceraldehyde-3-phosphate dehydrogenase coding genes. HIF-1 induced the expression of the above genes to enhance glycolysis to meet the needs of energy metabolism when the process of oxidative phosphorylation was inhibited by the condition of hypoxia[16-18].

The role of external hypoxia in initiating glycolysis and promoting its progression in tumor cells was previously uncertain. Past studies have investigated breast cancer cell lines MDA-mb-4 and MCF-7, which are highly and lowly malignant, respectively, cultured under normal oxygen conditions and found that the expression of HIF-1α and glycolytic enzymes and the level of glycolysis in MDA-mb-4 were greater in strength than those of MCF-7. Moreover, it is interesting that compared with the expression in normoxic environments, the expression of the genes in the breast cancer cell line MCF-7 under hypoxia was markedly elevated. However, there was no obvious change in MDA-mb-435, which suggested that the enhanced glycolysis in tumor cells was not caused by a single factor and that the environment and other factors were also involved in the process[19]. Renal carcinoma cell line RCC4, which has high expression of HIF-1α, was chosen for study because of its lack of the Von Hippel-Lindau gene (*vH-L* gene), which resulted in the blocked degradation of HIF-1α. In contrast with the level of glycolysis in normoxia and hypoxia conditions, no significant differences between these two conditions were found, but the level of glycolysis under normoxic conditions obviously decreased and increased clearly under hypoxic condition after restoring the regulatory ability of HIF-1α through the transfection of the *vH-L* gene in the RCC4 cell line[20]. It prompted the idea that the added HIF-1α protein accumulated or abnormally induced in cells might be the main factor in the regulatory process of glycolysis in tumor cells, and blocking HIF-1α might be an effective means of suppressing the glycolytic pathway in tumor cells.

Research has shown that most tumor tissues and their metastases have high expression of HIF-1α, and this is closely related to the glycolytic pathway of tumor cells[4]. We also confirmed the high expression of HIF-1α in esophageal cancer tissues in our previous studies[21,22]. To define the influence of hypoxia on the glycolytic level of esophageal carcinoma cells, we observed the changes in the expression of HIF-1α, the associated glycolytic proteins, such as GLUT-1, HK-Ⅱ, and LDHA, and the lactic acid content under different oxygen concentrations. The results showed that the expression of the proteins and the lactic acid concentration changed obviously under hypoxic conditions, and this suggests that the external oxygen concentration plays a regulatory role in glycolysis of esophageal carcinoma cells. Although there was a high glycolysis level under normal oxygen pressure in esophageal tumors, the glycolytic level could be further enhanced with a decrease in the external oxygen concentration. The enhanced glycolysis caused by hypoxia and the change in the genetic signaling

pathways by itself both evolved to the advantages of the Warburg effect. However, experiments also showed that HIF-1α and the associated glycolytic proteins, such as GLUT-1, HK-Ⅱ and LDHA, had a certain correlation in the enhanced process of glycolysis induced by hypoxia and played an important role in the pathophysiological mechanisms of promoting glycolysis, which was found to be enhanced in esophageal carcinoma cells. In addition to the involvement of glucose transport and glycolysis, the relevant proteins of glycolysis in tumor cells also included the glycolysis products, such as carbonic anhydrase (CA) and monocarboxylate transporters (MCTs). Studies have already confirmed that the *CAIX* and *MCT4* gene promoter sequences are the binding sites for HIF-1α, which can promote the transmembrane transport of lactic acid and hydrogen ions by enhancing the expression and activity of CAIX and MCT4 and ensure the smooth progress of glycolysis[23-26]. This finding suggests that HIF-1α, as a key factor in the process of regulating tumor glycolysis, may be involved in the enhanced glycolysis process of tumor cells through glucose transport, glycolysis activation and lactic acid transport.

To further confirm the impact of the high expression of HIF-1α on the process of glycolysis in esophageal tumors, we inhibited HIF-1α successfully in the cell lines Eca109 and TE13 using siRNA interference technology to observe the changes in the lactic acid concentration and the expression of the related glycolytic enzymes in the extracellular nutrient solution under different oxygen concentrations. The results showed that the mRNA and protein expression of GLUT-1 and HK-Ⅱ was down-regulated, and the lactic acid secretion reduced significantly, which was consistent with the trends found when HIF-1α was restrained, and compared with the control group under hypoxia, the expression was still at a low level, although the expression increased mildly when cultured under hypoxia. Thus, we speculate that the regulation of HIF-1α in the glycolysis of esophageal carcinomas depends on the activation of its downstream glycolytic enzymes. However, it is worth noting that the protein expression of LDHA changed slightly when HIF-1α was inhibited, and it also suggests that the regulation of LDHA was not only confined within the role of HIF-1α. When integrated with the literature, the regulation of glucose metabolism in tumors should be the consequence of a combination of multiple factors and multi-links with hypoxia. It also may be that HIF-1α is in a prominent position in the regulatory process, but not all of the glycolysis in esophageal cancers results from this single factor.

The PI3K/AKT signaling pathway is currently attracting much attention in cancer research, and it has been found that PI3K/AKT signaling is disordered in most human tumors and has been closely associated with proliferation, apoptosis, angiogenesis, invasion, metastasis, chemotherapy and radiotherapy resistance. The pathway plays an important role in adapting to the hypoxia environment and participates in the regulation of the HIF-1α singling pathway. A previous study reported that epidermal growth factor (EGF), fibroblast growth factor 2 and insulin-like growth factor 1 could induce the protein expression of HIF-1α by activating the PI3K/AKT signaling pathway and the corresponding tyrosine kinase receptors under normal oxygen pressure[7]. The mechanism of hypoxia tolerance activated by the PI3K/AKT pathway is widespread in mammalian cells, where HIF-1α exerts a critical intermediary role[27].

Based on the speculation that the PI3K/AKT signaling pathway might affect glycolysis in esophageal cancer cells, wortmannin, a specific ATP uncompetitive irreversible inhibitor of PI3K, with a half inhibitory concentration of 0.004 microns, was first extracted from the fungus *Penicillium wortmannin* in 1957. This compound was used to block the PI3K/AKT signaling pathway in the cell lines Eca109 and TE13. Wortmannin binds to PI3K gamma and causes the irreversible modification of lysine 833, while is the active site of PI3K[28]. Wortmannin at high concentrations also had different inhibitory effects on the PIKK family, such as mTOR, DNA-PK, ATM and ATR[29,30]. The results confirmed that the glycolytic level in esophageal cancer cells declined once the PI3K pathway was inhibited, regardless of whether the external oxygen supply was sufficient or not, and it was accompanied by a decrease in the expression of HIF-1α and the associated glycolytic proteins. This was not corrected effectively by culturing under hypoxia. Moreover, we found that the influence of wortmannin on the protein expression of the enzymes of glycolysis was different; the protein expression of GLUT-1 was down-regulated, whereas the mRNA expression did not change much by using wortmannin, which suggests that the regulatory effect of wortmannin on GLUT-1 might act at the protein level. In addition, the results also showed that the activities of HK-Ⅱ and LDH in cells and the secretion of lactic acid were restrained by adding wortmannin, and it could be elevated at different levels after treatment with hypoxia. Therefore, we surmise that the impact of the PI3K/AKT signaling pathway on glycolysis in esophageal tumors involves multiple steps and many factors, including the expression of key protein factors and the regulation of activities of different glycolytic enzymes. This suggests that the PI3K/AKT signaling pathway is involved into the process of glycolysis in esophageal carcinoma.

In summary, the level of glycolysis in esophageal carcinomas could be affected by hypoxic environment and is closely associated with the expression of HIF-1α. The PI3K/AKT pathway and HIF-1α are both involved in glycolysis in esophageal cancer cells, and LDHA, HK-Ⅱ and GLUT-1 might cause downstream effects. The regulation of glycolysis under hypoxic conditions could be achieved by activating the PI3K/AKT pathway through EGF and HIF-1α, and the ex-

pression level of glycolysis decreased markedly when the PI3K/AKT pathway was inhibited, which may be of potential therapeutic value in the future. This study will contribute to an effective treatment strategy for tumor cells under hypoxic environments and provide strong evidence and new thoughts on the diagnosis and treatment of cancers. It also provides direction on the development of new antitumor drugs and thus gives more reasonable and individualized therapeutic schedules to cancer patients in the clinic.

COMMENTS

Background

Esophageal cancer is the sixth leading cause of cancer-related death in China. Although recent developments in therapeutic strategies have helped cure many patients with early-stage disease, the prognosis of patients with advanced disease and metastasis remains poor.

Research frontiers

Tumor hypoxia is an important factor leading to radiotherapy and chemotherapy resistance and promotes tumor invasion and metastasis. It also involves cell energy metabolism. Hypoxia-inducible factor-1 alpha (HIF-1α) is a key regulatory factor of tissue adaptation under hypoxia and is highly expressed in most tumors and metastases. It is also inseparable from the glycolytic pathway of tumor cells. The PI3K/AKT signaling pathway widely exists in cells and is involved in cell growth, proliferation, differentiation and regulatory signal transduction pathways. It is also one of the most closely regulatory pathways of the glucose metabolism of cancer cells besides participating in the regulation of HIF-1α expression.

Innovations and breakthroughs

The authors investigated the signaling pathway of glycolysis from the perspective of hypoxia tolerance in esophageal tumor cells based on HIF-1α and analyzed multiple related glycolytic genes through the PI3K/AKT-HIF-1α pathway to obtain more information regarding the molecular regulatory mechanism of esophageal cancers.

Applications

The findings of this study indicated that the PI3K/AKT pathway and HIF-1α are both involved in the process of glycolysis in esophageal cancer cells, and the regulation of glycolysis in the hypoxia condition could be achieved by activating the PI3K/AKT pathway through EGF and HIF-1α. The expression level of glycolytic enzymes decreased markedly when the PI3K/AKT pathway was inhibited.

Terminology

The study adopted mature and reliable laboratory methods, such as fluorescence analysis, spectrophotometry, real time PCR, Western blot and siRNA interference technology.

Peer-review

Poor prognosis of esophageal cancer is a well-known fact and early diagnosis of this disease is so important. This is a well-written paper.

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ORIGINAL ARTICLE

Basic Study

miR-29a up-regulation in AR42J cells contributes to apoptosis *via* targeting *TNFRSF1A* gene

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Author contributions: Fu Q and Chen L performed the majority of experiments; Qin T and Zhang HW designed the research and provided financial support for this work; Liu CJ conducted the experimental analysis; Wang YZ and Hu MX provided vital reagents and were involved in editing the manuscript; Zhang X and Chu HY analyzed sequencing data and developed analysis tools; Fu Q, Qin T and Chen L wrote the paper.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of People's Hospital of Zhengzhou University.

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Ethics Committee of People's Hospital of Zhengzhou University.

Conflict-of-interest statement: We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any products, service and/ or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled "miR-29a up-regulation in the AR42J cells contributes to apoptosis via targeting the TNFRSF1A gene".

Data sharing statement: No additional data are available.

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Abstract

AIM: To investigate the expression of miR-29a in rat acute pancreatitis and its functional role in AR42J cell apoptosis.

METHODS: Twelve SD rats were divided into a control group and an acute edematous pancreatitis (AEP) group randomly. AEP was induced by intraperitoneal injection of L-arginine (150 mg/kg) in the AEP group and equal volume of 0.9% NaCl was injected in the control group. The apoptosis of acinar cells in pancreatic tissue was determined by TUNEL assay. miRNA chip assay was performed to examine the expression of miRNAs in two groups. Besides, to further explore the role of miR-29a in apoptosis *in vitro*, recombinant rat TNF-α (50 ng/mL) was administered to treat the rat pancreatic acinar cell line AR42J for inducing AR42J cell apoptosis. Quantitative real-time PCR (qRT-PCR) was adopted to measure miR-29a expression. Then, miRNA mimic, miRNA antisense oligonucleotide (AMO) and control vector were used to transfect AR42J cells. The expression of miR-29a was confirmed by qRT-PCR and

the apoptosis rate of AR42J cells was detected by flow cytometry analysis. Western blot was used to detect the expression of activated caspase3. Moreover, we used bioinformatics software and luciferase assay to test whether TNFRSF1A was the target gene of miR-29a. After transfection, qRT-PCR and Western blot was used to detect the expression of TNFRSF1A in AR42J cells after transfection.

RESULTS: The expression of miR-29a was much higher in the AEP group compared with the control group as displayed by the miRNA chip assay. After inducing apoptosis of AR42J cells *in vitro*, the expression of miR-29a was significantly increased by 1.49 ± 0.04 times in comparison with the control group. As revealed by qRT-PCR assay, the expression of miR-29a was 2.68 ± 0.56 times higher in the miR-29a mimic group relative to the control vector group, accompanied with an obviously increased acinar cell apoptosis rate (42.83 ± 1.25 *vs* 24.97 ± 0.15, $P < 0.05$). Moreover, the expression of miR-29a in the miRNA AMO group was 0.46 ± 0.05 times lower than the control vector group, and the cell apoptosis rate was much lower accordingly (17.27 ± 1.36 *vs* 24.97 ± 0.15, $P < 0.05$). The results of bioinformatics software and luciferase assay showed that TNFRSF1A might be a target gene of miR-29a. *TNFRSF1A* expression was up-regulated in the miR-29a mimic group, while the miR-29a AMO group showed the reverse trend.

CONCLUSION: miR-29a might promote the apoptosis of AR42J cells *via* up-regulating the expression of its target gene *TNFRSF1A*.

Key words: Acute edematous pancreatitis; miR-29a; Apoptosis; AR42J; Target gene; *TNFRSF1A*

Core tip: Apoptosis is a self-protection mechanism in acute pancreatitis. miRNAs are short non-coding RNAs and play important roles in regulating gene expression in multiple cellular processes, such as apoptosis. Here, our group studied the role of miR-29a in pancreatic acinar cell apoptosis. The pancreatic acinar cells showed a tendency to apoptosis when the expression of miR-29a elevated, while the apoptosis rate exhibited the opposite trend by down-regulating the expression of miR-29a. Moreover, we found TNFRSF1A, which encode TNFR1 protein, was a target gene of miR-29a. Our results demonstrated that miR-29a could promote the apoptosis of pancreatic acinar cells *via* up-regulating the expression of *TNFRSF1A* gene in acute pancreatitis.

Fu Q, Qin T, Chen L, Liu CJ, Zhang X, Wang YZ, Hu MX, Chu HY, Zhang HW. miR-29a up-regulation in AR42J cells contributes to apoptosis *via* targeting *TNFRSF1A* gene. *World J Gastroenterol* 2016; 22(20): 4881-4890 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i20/4881.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i20.4881

INTRODUCTION

Tumor necrosis factor (TNF)-α is a pleiotropic cytokine that plays a crucial role in angiogenesis, inflammation, proliferation and apoptotic cell death[1]. TNF-α is involved in the development of pancreatitis, and mediates apoptosis in acinar cell suspensions *in vitro* as well as in an *in vivo* model of pancreatitis. Pancreatic acinar cells produce, release, and respond to TNF-α[2], which acts by binding to its two receptors, TNF-R1 and TNF-R2, on the cell surface. TNF-R1, which is the major signaling receptor for TNF-α, is expressed on all cell types. Both soluble and membrane-bound forms of the cytokine can activate TNFR1. The binding of TNF to TNFR1 results in immediate nuclear factor-κB (NF-κB) activation and subsequent apoptosis[3,4].

MicroRNAs (miRNAs) are short non-coding RNAs involved in multiple cellular processes including development, proliferation, differentiation, apoptosis and metabolism[5]. Most studies on miRNAs have focused on the repression of their target genes by binding to complementary sites, causing target mRNAs degradation and/or translational repression[6-9]. In recent years, it had been demonstrated that miRNAs could up-regulate the expression of their target genes. Vasudevan *et al*[10] demonstrated that human miRNA369-3 directs association of the proteins with AREs (AU-rich elements) to activate translation, while let-7 and the synthetic miRNA miRNAcxcr4 induced upregulation of target genes. miR-29a has been extensively demonstrated to play an important role in apoptosis[11-15]. Using miRNA microarray analysis, we found that miR-29a was elevated in a rat model of AEP *in vivo*. TNFRSF1A, which encodes the TNFR1 protein, was predicted to be the target gene of miR-29a with the aid of three online programs (TargetScan, miRanda and TarBase). Our results demonstrated that miR-29a is elevated significantly in AEP *in vivo*. The rate of apoptosis and TNFRSF1A expression increased significantly in AR42J cells following upregulation of miR-29a expression.

MATERIALS AND METHODS

Animal care

All animal experiments were approved by the Animal Research Ethics Committee of People's Hospital of Zhengzhou University, Zhengzhou, China. Surgery was performed under chloral hydrate anesthesia and all efforts were made to minimize animal suffering.

In vivo AEP model

Twelve male SD rats (250-300 g) were divided into two groups randomly ($n = 6$). All rats were anesthetized with 10% chloral hydrate (300 mg/kg,

Table 1 Sequences of miR-29a mimic and antisense oligonucleotide

	Base sequence
miR-29a mimic	ACCCCTTAGAGGATGACTGATTTCTTTTGGTGTTCAGAGTCAATAGAATTTTCTAGCACCATCTGAAATCGGTTATAATGATTGGGGA
miR-29a AMO	ACTGATTTCTTTTGGTGTTCAG

i.p.). 150 mg/kg L-arginine (Sigma, United States) was injected intraperitoneally to establish the AEP model *in vivo*. The rats in the control group were injected with equivalent saline. After 12 h the pancreas of the rats was removed.

TUNEL assay

The apoptosis of acinar cells in pancreatic tissue was determined by terminal-deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL) assay by using the *in situ* cell death detection kit (Promega, China). According to the manufacturer's instructions, the tissue was fixed in 10% buffered formaldehyde, embedded in paraffin, and 4-μm sections were adhered to glass slides. After dewaxing and rehydration, the sections were incubated with TUNEL reaction mixture at 37 ℃ for 1 h. Finally, the sections were analyzed under a fluorescence microscope (Olympus, Tokyo, Japan). TUNEL-positive cells displayed brown fluorescence.

miRNA microarray

Total RNA of pancreas tissue was extracted using TRIzol. miRNA microarray analysis was applied to detect the differential expression of miRNAs in pancreas tissue between the two groups.

miR-29a mimic and antisense oligonucleotide construct

The mimic and antisense oligonucleotide of miR-29a, which were designed and synthesized chemically with the help of Genechem Bio Company (Shanghai, China), were inserted into a lentiviral vector carrying the green fluorescent protein (*GFP*) gene. The sequences of miR-29a mimic and AMO are presented in Table 1.

Cell culture and lentiviral transfection

The AR42J cell line (rat pancreatic acinar cell, Institute of Shanghai Cell Biology, Shanghai, China) was cultured in DMEM-F12 medium (Gibco, United States) containing 20% fetal bovine serum (FBS) (Gibco, United States) in a humidified incubator at 37 ℃ with an atmosphere of 5% CO_2. The AR42J cells (1×10^6 cells/well) were seeded in 6-well plates 24 h before transfection and infected at an MOI of 50 with 10 μg/mL of polybrene for 12 h. After 12 h, the transfection liquid was removed and 2 mL normal medium was added for continued culturing. Seventy-two hours after transfection, green fluorescence was observed under a fluorescence microscope.

Induction of apoptosis and amylase assay

AR42J cells at 1×10^6/well were seeded into 6-well plates. After 24 h, the medium was removed and DMEM-F12 medium containing 50 ng/mL recombinant rat TNF-α (Peprotech, United States) was added. Twelve hours later, the supernatant was collected and centrifuged at 1000 rpm for 5 min and then used to detect the level of amylase using an amylase kit (Jiancheng Bio, Nanjing, China) according to the manufacturer's instructions. The control group was incubated with DMEM-F12 medium only.

Western blot analysis

Total protein from the cultured AR42J cells was extracted with RIPA lysis buffer (150 mmol/L NaCl, 50 mmol/L Tris–HCl (pH 7.4), 1% NP-40, 1 μg/mL leupeptin, 1 mmol/L deoxycholic acid and 1 mmol/L EDTA) containing 1 mmol/L phenylmethylsulfonyl fluoride, according to the manufacturer's instructions (Beyotime Bio, Wuhan, China). Proteins (40 μg) from each sample were loaded and separated on a 12% SDS polyacrylamide gel. Proteins were then electrophoretically transferred onto PVDF membranes (Millipore, Bedford, MA, United States), which were then incubated with diluted anti-rat monoclonal activated caspase3 antibody at 1/1000 (CST, United States), anti-rat TNFR1 at 1/200 (SANTA CRUZ Bio, United States) or anti-β-actin at 1/1000 (CST, United States) at 4 ℃ overnight. On the following day, membranes were incubated with an HRP secondary antibody (1:5000) at 37 ℃ for 2 h and then signals were visualized with an electrochemiluminescence kit (Pierce, Rockford, IL, United States).

Flow cytometry analysis of apoptosis

AR42J cells were seeded into 6-well plates (5×10^5/well) and incubated with DMEM-F12 medium containing 50 ng/mL recombinant rat TNF-α for 24 h. Cells were harvested, washed twice with 1 × PBS and then stained using an annexin V-APC apoptosis kit (KeyGEN Bio, Nanjing, China) according to the manufacturer's instructions. All flow cytometric analyses were carried using a FACS Caliber flow cytometer (San Jose, CA, United States).

Target prediction

Three online programs, TargetScan (http://www.targetscan.org), miRanda (http://www.microrna.org/microrna/home.do), and TarBase (http://diana.cslab.ece.ntua.gr/tarbase), were used in combination for predicting the target genes of miR-29a.

Luciferase reporter assay

HEK 293T cells (Institute of Shanghai Cell Biology, Shanghai, China) were cultured in DMEM medium

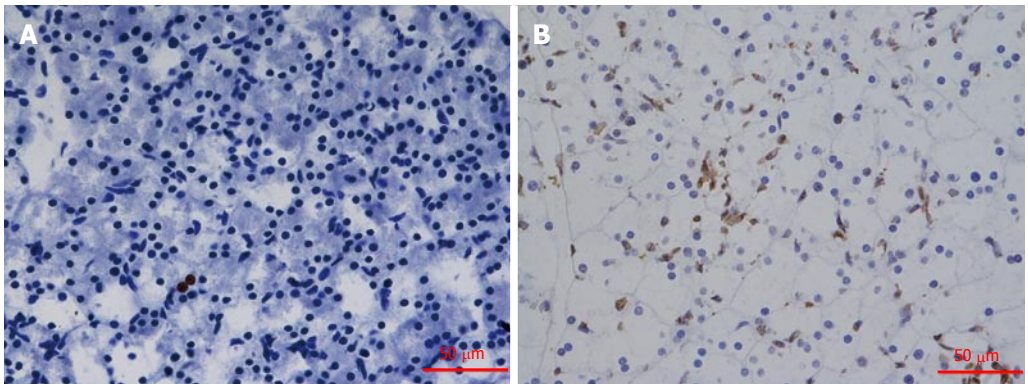

Figure 1 TUNEL staining of pancreatic tissue (× 400). TUNEL-positive cells displayed brown fluorescence. A: TUNEL staining was detected in control rats; B: The tissue of the L-arginine treated rats. The apoptosis increased significantly in Figure 1B.

(Gibco, United States) containing 10% FBS (Gibco, United States) in a humidified incubator at 37 ℃ under 5% CO_2. HEK 293T cells were seeded in 24-well plates 24 h before transfection with 100 ng psiCHECK^{TM}-2 vector (RiboBio, Guangzhou, China) containing the wild-type TNFRSF1A 3'UTR (designated TNFRSF1A 3'UTR-WT) or the TNFRSF1A mutant (designated TNFRSF1A 3'UTR -Mut) together with 50 nmol/L miR-29a miRNA mimic; a non-target control was used in the control group. Luciferase activity was measured 48 h after transfection. Lipofectamine 2000 transfection reagent (Invitrogen, United States) was used for co-transfection of RNA oligonucleotides and plasmids.

Quantitative real-time RT-PCR

Total RNA was isolated using Trizol (Invitrogen, United States) and then reverse-transcribed into cDNA with PrimeScript RT Master Mix (Takara, Japan) according to the manufacturer's instructions. Quantitative real-time PCR was performed using the SYBR Premix Ex Taq^{TM} kit (Takara, Japan). Specific primer sequences are listed as follows: 5'-GTGCTGTTGCCTCTGGTTATCT-3' (forward) and 5'-GAGACAGGATGACTGAAGCGTG-3' (reverse) for *TNFRSF1A*; 5'-TTCAACGGCACAGTCAAGG-3' (forward) and 5'-CTCAGCACCAGCATCACC-3' (reverse) for *GAPDH*. The primers for miR-29a and U6 were synthesized by Guangzhou RiboBio Co. Ltd. (China). Expression of *TNFRSF1A*, relative to *GAPDH* and expression of miR-29a, relative to U6, were determined using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

The results are expressed as mean ± SD from at least three separate experiments. Statistical analyses were performed using SPSS 13.0 software and comparisons were made using Student's *t*-test and one-way ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

TUNEL assay

Apoptosis of pancreatic acinar cells was determined by TUNEL assay (Figure 1). The results of TUNEL assays showed that the apoptosis of pancreatic acinar cells increased significantly in the L-arginine group compared with that in the control group ($P < 0.05$).

miR-29a expression in the AEP model in vivo

miRNA-microarray analysis of the miRNAs in the rat pancreas was performed to compare the expression of miRNAs between the control and AEP groups. Numerous miRNAs were significantly different in the AEP group compared with the control group (Figure 2). The expression level of miR-29a was much higher in the AEP group compared with the control group ($P < 0.01$).

AR42J cell apoptosis in vitro

As shown in Figure 3A, the level of amylase in the experimental group increased significantly compared with that in the control group at 12 h after exposure of AR42J cells to the TNF-α ($P = 0.042$). Activated caspase 3 was detected by Western blot analysis as described. Caspase 3 expression increased obviously after the AR42J cells were exposed to TNF-α for 12 h. The apoptosis rate was significantly higher (6.26-fold) in the experimental group compared with that in the control group ($P = 0.026$; Figure 3C). These results demonstrated that the AEP model was successfully established *in vitro*.

Expression of miR-29a is increased in vitro

The expression level of miR-29a was confirmed by quantitative real-time RT-PCR. As shown in Figure 4, the level of miR-29a was significantly higher (1.49-fold) in the experimental group compared with that in the control group after AR42J cells were exposed to TNF-α for 3 h ($P = 0.034$).

miR-29a expression after lentiviral transfection

The lentiviral vector carrying the miR-29a mimic or AMO was transfected into the AR42J cells as described. After 72 h, the cells were evaluated for expression of green fluorescence under a fluorescence

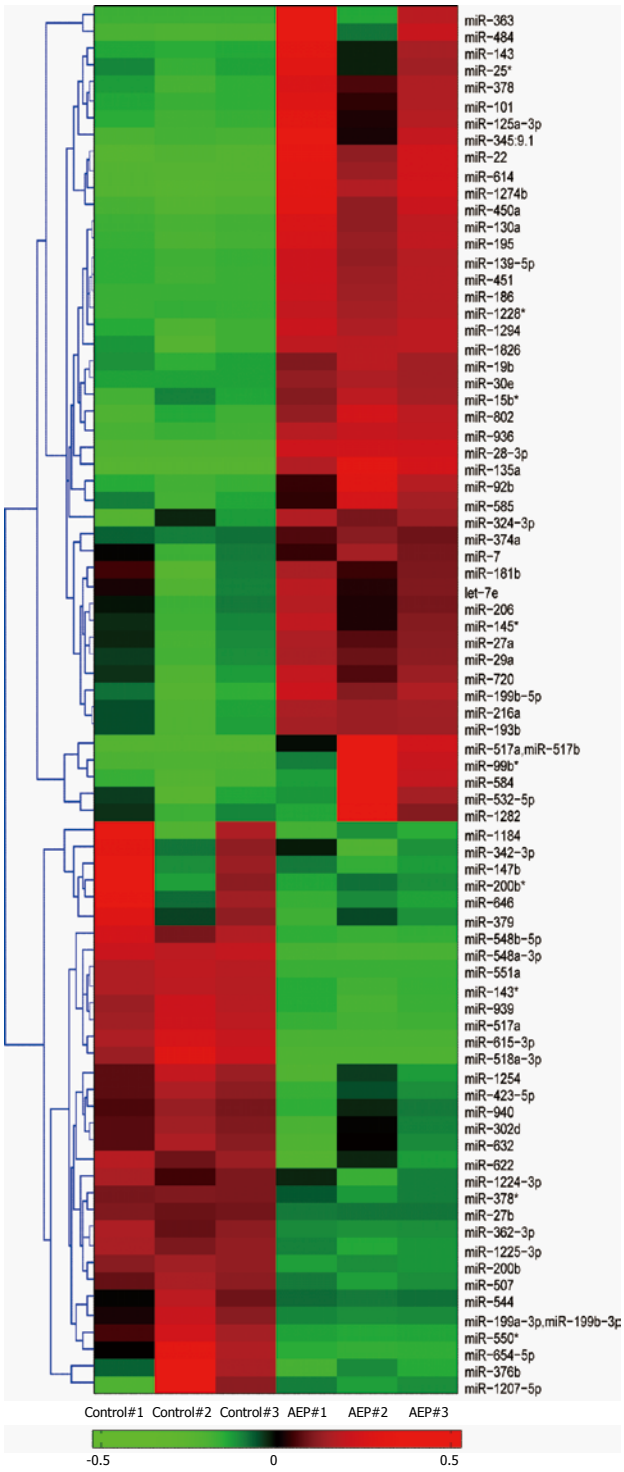

Figure 2 Hierarchically clustered heat map illustrating the changes in miRNA expression profiles between the acute edematous pancreatitis groups and control groups. The significantly expressed miRNA clusters were identified using the Student's *t*-test. The red and green sections represent an increase and a decrease in miRNA expression, respectively, between control group and AEP group. The expression of miR-29a was significantly up-regulated in the AEP group compared with the control group. AEP: Acute edematous pancreatitis.

microscope. As shown in Figure 5A, most of the AR42J cells expressed green fluorescence, which indicated that the AR42J cells were successfully transfected by the lentivirus, from which protein was successfully expressed. miR-29a expression by lentivirus-transfected AR42J cells was analyzed by qRT-PCR. Cells transfected with the miR-2a mimic expressed significantly increased miR-29a levels (P = 0.018; Figure 5B), while cells transfected with the miR-29a AMO expressed lower miR-29a levels (P = 0.020; Figure 5B) compared with the vehicle groups.

Induction of apoptosis after transfection

After lentiviral transfection, the AR42J cells were exposed to TNF-α and the level of amylase in the supernatant was detected as described, suggesting that the AEP model was established successfully (Figure 6A). To investigate the proapoptotic activity of miR-29a, we detected the rate of apoptosis and activated caspase 3 expression in AR42J cells after the AEP model was established *in vitro*. Interestingly, compared with the vehicle group, upregulation of miR-29a increased the expression of activated caspase 3, while the miR-29a AMO group showed a lower level of activated caspase 3 (Figure 6B). The apoptosis rate in the miR-29a mimic group (42.83 ± 1.25%) was significantly higher than that in the vehicle group (24.97 ± 0.15%) (P = 0.030; Figure 6C). In contrast, the apoptosis rate in the miR-29a AMO group (17.27 ± 1.36%) was significantly lower than that in the vehicle group (P = 0.025; Figure 6C).

TNFRSF1A is regulated by miR-29a

By using three online programs (TargetScan, miRanda and TarBase), we predicted that TNFRSF1A was the target gene of miR-29a. As shown in Figure 7, the 3′ UTR of *TNFRSF1A* contains a putative target site for miR-29a. To obtain direct evidence in support of this prediction, the 3′UTR of the rat TNFRSF1A gene was cloned into the *Xba* Ⅰ -site of the pGL3-luciferase reporter vector, which was then used to test its capacity to serve as the direct functional target of miR-29a; the construct was designated pGL3-TNFRSF1A-WT. In parallel, another luciferase reporter construct was prepared in which the putative miR-29a targeting region was specifically mutated and predicted to abolish miR-29a binding; this construct was designated pGL-TNFRSF1A-Mut. Transient transfection of HEK293T cells with pGL-TNFRSF1A-wt and miR-29a led to a significant decrease in luciferase activity compared to that in the control group (P = 0.0018). The activity of the mutant reporter construct, however, was unaffected by co-transfection with miR-29a (P = 0.626).

Expression of TNFRSF1A after transfection

To examine the effect of miR-29a on TNFRSF1A expression, AR42J cells were transfected with a miR-29a mimic or an AMO. TNFRSF1A mRNA levels were analyzed by qRT-PCR. As shown in Figure 8A, the expression of TNFRSF1A was significantly higher (1.86-fold) in the miR-29a mimic group compared with the vehicle group (P = 0.022), while the expression

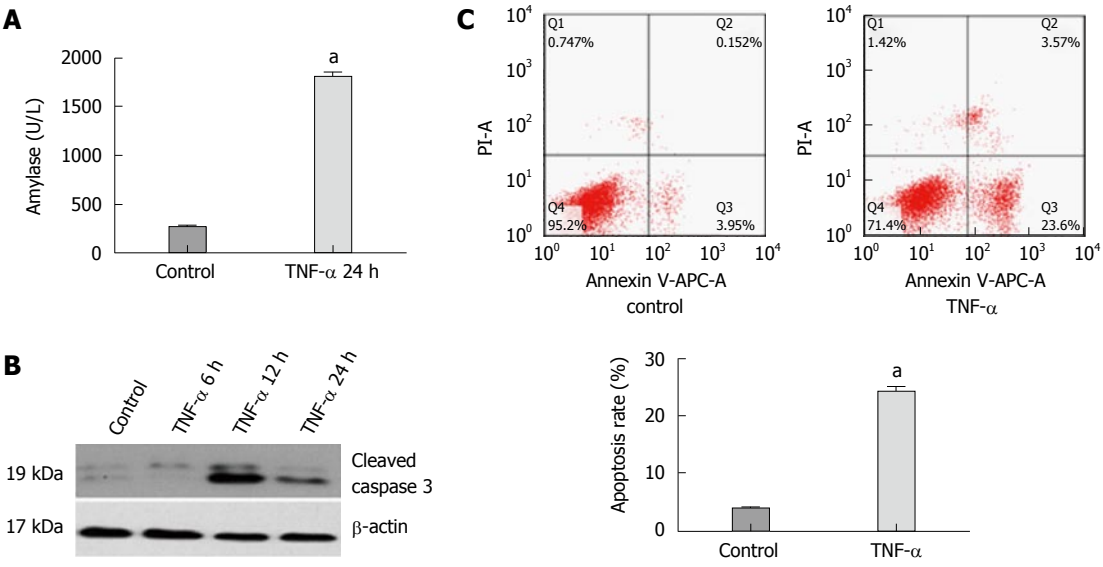

Figure 3 Expression of amylase, activated caspase 3 protein, apoptosis rate of AR42J cells and miR-29a level increase in the experimental group compared with the control group. A: The expression of amylase analysis in the supernatant; B: Western blot analysis of activated caspase 3 in AR42J cells; C: The apoptosis rate of AR42J cells after the treatment with TNF-α for 24 h. Date were obtained from three independent experiments in triplicate and are shown as the mean ± SD. $^{a}P < 0.05$ *vs* control group.

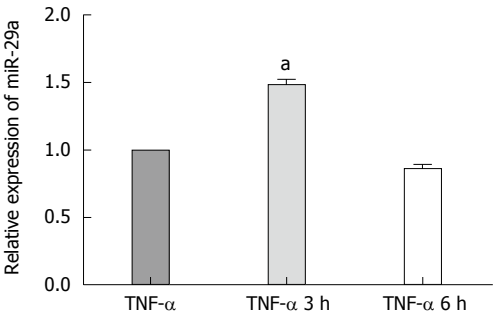

Figure 4 Quantitative real-time PCR analysis of miR-29a in AR42J cells at 3 h and 6 h. The expression of miR-29a was normalized to U6 expression using $2^{-\Delta\Delta ct}$. Data were obtained from three independent experiments in triplicate and are shown as the mean ± SD. $^{a}P < 0.05$ *vs* control group.

was significantly lower (0.61-fold) in the miR-29a AMO group ($P = 0.048$). In parallel, we analyzed the expression of TNFR1 protein in the miR-29a mimic and AMO groups. The miR-29a mimic group expressed higher levels of TNFR1 protein compared with the vehicle group, while the levels were lower in the miR-29a AMO group (Figure 8B).

DISCUSSION

AP is a common clinical condition with high morbidity and mortality, and its incidence increases over recent years[16,17]. There are two patterns of pancreatic acinar cells death: necrosis and apoptosis. Apoptosis is a physiological and programmed form of cell death. The stereotypical and characteristic morphology of apoptosis includes cell shrinkage, retention of organelles and nuclear chromatin condensation, which occurs in response to stimuli[18]. The relationship between apoptosis and AP had been extensively investigated and it has been demonstrated that the severity of AP is inversely related to the rate of apoptosis and correlates directly with the extent of necrosis[19,20]. The feature of the AP model which is induced by L-arginine is reproducible and dose-dependent. In this study, we successfully induced AEP with L-arginine (150 mg/kg) *in vivo* and the result of TUNEL assays confirmed that apoptosis occurred in pancreatic acinar cells.

The AR42J cell line used in this study possessed the properties of exocrine digestive enzymes[21]. There are many advantages of AR42J cells for the investigation of signaling mechanism in the pancreas, such as the ease of cell line culture maintenance, high transduction efficiency and responsiveness to many agonists[22]. The AR42J cell line had been used extensively to establish the AP model *in vitro*. Chanthaphavong *et al*[23] demonstrated that TNF-α induced apoptosis of several cell lines. Furthermore, a recent study showed that TNF-α activated the NF-κB pathway and induced the expression of proinflammatory mediators in pancreatic acinar cells[24]. The results of our study demonstrated that rat recombinant TNF-α cytokine was used successfully to establish the AEP model *in vitro*.

miRNAs are short non-coding RNAs that play an important role in regulating gene expression in multiple cellular processes including apoptosis, metabolism, proliferation, differentiation and development[5]. To date, over 15000 mature miRNAs have been identified in 133 species[25]. In recent years, it has been demonstrated that miRNAs can upregulate the target genes. Vasudevan *et al*[10] showed that human miRNA369-3 directed association of the proteins with AREs to activate translation, and let-7 and the synthetic miRNA miRNAcxcr4 could induce up-regulation of target genes.

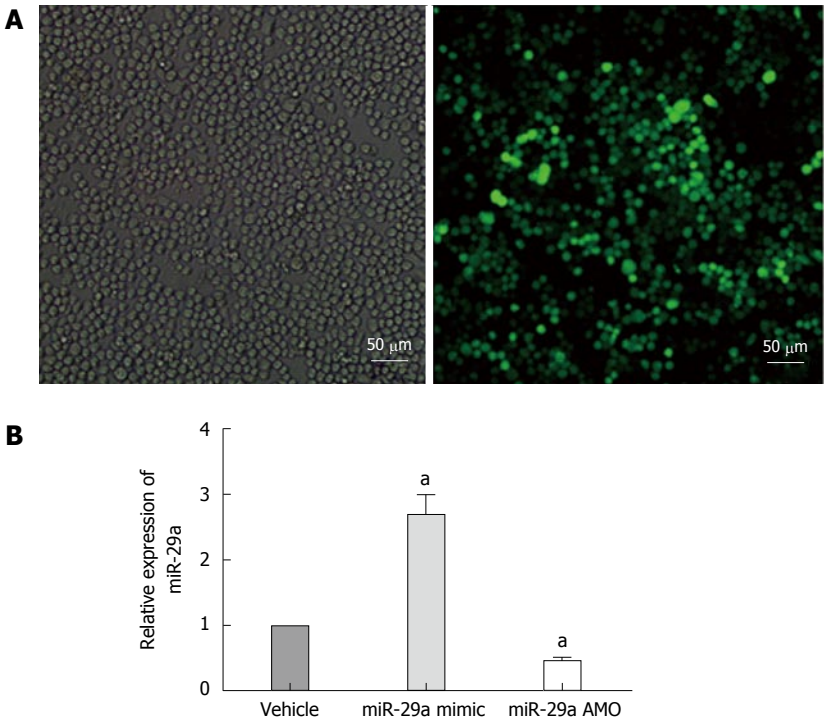

Figure 5 Lentiviral transfection and miRNA expression after transfection. A: Cells were infected with 50 MOI of lentivirus, and imaged 72 h post-transfection. Comparison of bright field filter view to FITC filter view (GFP-expression cells) for the same fields of cells showed about 90% infection efficiency by 72 h; B: Quantitative real-time PCR analysis of miR-29a expression in the AR42J cells after transfection. Date are shown as a ratio of mi-29a mimic and AMO groups to vehicle groups using the $2^{-\Delta\Delta ct}$. Data are representative of three independent experiments. $^{a}P < 0.05$ *vs* vehicle group.

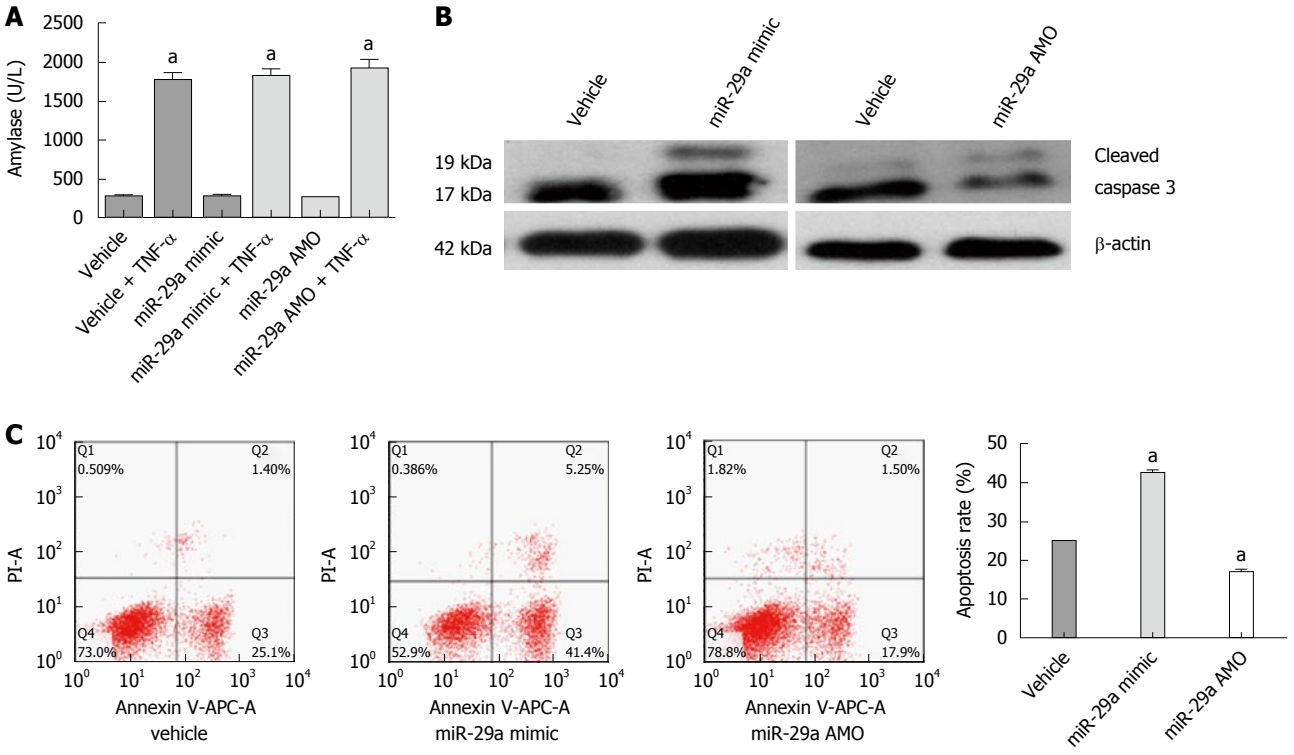

Figure 6 miR-29a promotes the apoptosis of the AR42J cells. A: The amylase analysis in the supernatant increased obviously; B: Western blot analysis of activated caspase 3 in AR42J cells; C: The apoptosis rate of AR42J cells was determined by FACS analysis. Data are representative of mean ± SD from three independent experiments performed in triplicate. $^{a}P < 0.05$ *vs* control or vehicle group.

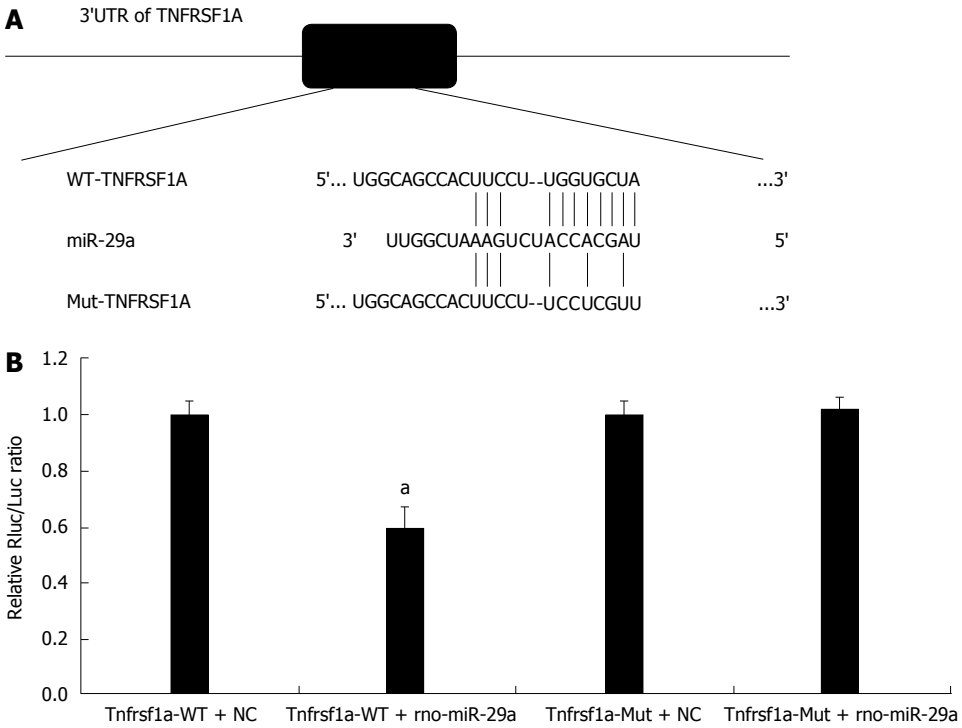

Figure 7 miR-29a targets *TNFRSF1A*. A: The predicted miR-29a binding sites within the 3'UTR of *TNFRSF1A* and mutant version generated by site mutagenesis are shown; B: Luciferase activity was determined 48 h after transfection. The ratio of normalized sensor to control luciferase activity is shown. Data are shown as the mean ± SD and were obtained from three independent experiments performed in triplicate ($^aP < 0.05$ *vs* control miR-transfected cells).

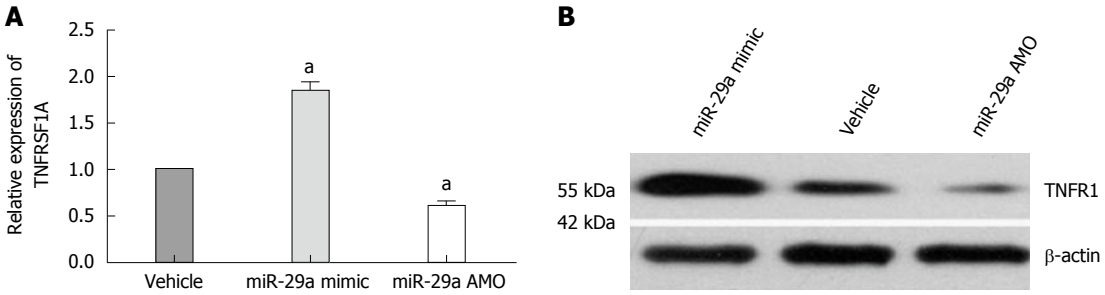

Figure 8 miR-29a promotes *TNFRSF1A* gene expression. A: Quantitative real-time RT-PCR analysis of TNFRSF1A expression in AR42J cells after transfection. Date are shown as a ratio of miR-29a mimic and AMO groups to vehicle group using the $2^{-\Delta\Delta ct}$. Data are representative of three independent experiments ($^aP < 0.05$ *vs* vehicle group); B: Western blot analysis of TNFR1 protein in AR42J cells after transfection.

miR-29a has been extensively demonstrated to promote cell apoptosis *via* suppressing survival genes. Direct repression of CDC42 and p85α by miR-29a can result in the activation of p53 and induction of apoptosis[11]. MCL1, which encodes an anti-apoptotic Bcl-2 family protein, is also the target gene of miR-29a. By repressing MCL-1, miR-29a sensitizes cholangicarcinoma and ALT$^+$ ALCL cells to apoptosis[14]. The results of our miRNA microarray and qRT-PCR analyses revealed that miR-29a is elevated in the AEP model *in vivo* and *in vitro*. miR-29a mimic and AMO were designed to investigate the function of miR-29a. This technology utilizes non-natural synthetic nucleic acids, which bind to the unique sequence of the target mRNAs in a gene-specific manner and has the same effects as the endogenous miRNAs[26,27]. Our result showed that AR42J cells showed a tendency to apoptosis in the miR-29 mimic group, while the apoptosis rate was significantly decreased in the miR-29a AMO group in the AEP model *in vivo*. *TNFRSF1A*, which encodes the TNFR1 protein, was predicted to be the target gene of miR-29a with the help of three online programs (TargetScan, miRanda and TarBase). TNF-R1, which is the major signaling receptor for TNF-α, is expressed on all cell types. Both soluble and membrane-bound forms of the cytokine can activate TNFR1. The binding of TNF-α to TNFR1 results in immediate NF-κB activation and subsequent apoptosis[3,4]. Our study demonstrated that miR-29a mediated up-regulation of the *TNFRSF1A* gene. AR42J cells transfected with the miR-29a mimic expressed higher levels of *TNFRSF1A* mRNA and TNFR1 protein, while the miR-29a AMO group exhibited the opposite trend. miR-29a promotes the expression of *TNFRSF1A*

gene and its regulation mechanism is not binding to and degrading *TNFRSF1A* gene as the the result of luciferase assay in HEK 293T cells. We hypothesized that the combination of miR-29a and TNFRSF1A gene might guide some protein factors, which promote the transcription and translation of genes in pancreatic acinar cells, bind to the target gene. Further investigations need to be performed.

In summary, miR-29a is elevated significantly in AEP, indicating that miR-29a might promote pancreatic acinar cell apoptosis by enhancing expression of *TNFRSF1A* gene.

COMMENTS

Background

Apoptosis is a self-protective mechanism in acute pancreatitis. miRNAs are short non-coding RNAs involved in multiple cellular processes including development, proliferation, differentiation, metabolism and apoptosis.

Research frontiers

It has been reported that miR-29a plays an important role in apoptosis, but little is known about the effect of miR-29a on apoptosis in acute pancreatitis and its regulatory mechanisms.

Innovations and breakthroughs

In this study, the authors demonstrated that miR-29a is elevated significantly in acute edematous pancreatitis. The rate of apoptosis and TNFRSF1A expression increased significantly in AR42J cells following upregulation of miR-29a expression. miR-29a promotes pancreatic acinar cell apoptosis by enhancing expression of *TNFRSF1A* gene directly.

Applications

The study results suggest that miR-29a promotes pancreatic acinar cell apoptosis by enhancing expression of *TNFRSF1A* gene directly in acute pancreatitis, and these findings may provide a theoretical basis for the prevention and treatment of acute pancreatitis.

Terminology

MicroRNAs (miRNAs) are a set of 21- to 24- nucleotide(nt), endogenous, non-coding, regulatory RNA molecules that contribute to modulating the expression levels of specific proteins based on base pairing with their target mRNA molecules.

Peer-review

Authors demonstrated that miR-29a, which has been demonstrated to play an important role in apoptosis, is elevated significantly in acute edematous pancreatitis. The data are interesting and the experiments are well organized. Those findings give novel information on the pathogenesis of acute pancreatitis.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Rectal cancer staging: Multidetector-row computed tomography diagnostic accuracy in assessment of mesorectal fascia invasion

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Informed consent statement: Every patient gave his informed consent, as required by our Institution.

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Abstract

AIM: To assess the diagnostic accuracy of multidetector-row computed tomography (MDCT) as compared with conventional magnetic resonance imaging (MRI), in identifying mesorectal fascia (MRF) invasion in rectal cancer patients.

METHODS: Ninety-one patients with biopsy proven rectal adenocarcinoma referred for thoracic and abdominal CT staging were enrolled in this study. The contrast-enhanced MDCT scans were performed on a 256 row scanner (ICT, Philips) with the following acquisition parameters: tube voltage 120 KV, tube current 150-300 mAs. Imaging data were reviewed as axial and as multiplanar reconstructions (MPRs) images along the rectal tumor axis. MRI study, performed on 1.5 T with dedicated phased array multicoil, included multiplanar T2 and axial T1 sequences and diffusion weighted images (DWI). Axial and MPR CT images independently were compared to MRI and MRF involvement was determined. Diagnostic accuracy of both modalities was compared and statistically analyzed.

RESULTS: According to MRI, the MRF was involved in 51 patients and not involved in 40 patients. DWI allowed to recognize the tumor as a focal mass with high signal intensity on high b-value images, compared with the signal of the normal adjacent rectal wall or with the lower tissue signal intensity background. The number of patients correctly staged by the native axial CT images was 71 out of 91 (41 with involved MRF; 30 with not involved MRF), while by using the MPR 80 patients were correctly staged (45 with involved MRF; 35 with not involved MRF). Local tumor staging suggested by MDCT agreed with those of MRI, obtaining for CT axial images sensitivity and specificity of 80.4% and 75%, positive predictive value (PPV) 80.4%, negative predictive value (NPV) 75% and accuracy 78%; while performing MPR the sensitivity and specificity increased to 88% and 87.5%, PPV was 90%, NPV 85.36% and accuracy 88%. MPR images showed higher diagnostic accuracy, in terms of MRF involvement, than native axial images, as compared to the reference magnetic resonance images. The difference in accuracy was statistically significant (P = 0.02).

CONCLUSION: New generation CT scanner, using high resolution MPR images, represents a reliable diagnostic tool in assessment of loco-regional and whole body staging of advanced rectal cancer, especially in patients with MRI contraindications.

Key words: Magnetic resonance; Multi detector computed tomography; Rectal cancer; Mesorectal fascia; Multiplanar reconstructions

Core tip: The introduction of new generation of multidetector-row computed tomography (MDCT) scanner allowed thin-collimation scanning and high spatial resolution, resulting in improved multiplanar reconstructions (MPRs) and could be potentially useful, in a single examination, for local staging and distant metastases evaluation in rectal cancer patients. On these basis in our study we assessed the accuracy of high row number MDCT for the prediction of tumor invasion of the mesorectal fascia, being MRI findings as reference standard, and whether the addition of high-resolution MPR images can provide greater accuracy.

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INTRODUCTION

Treatment options in rectal cancer patients, such as total mesorectal excision (TME) and preoperative neoadjuvant radiochemotherapy in advanced tumor stages[1,2], have greatly increased the importance of accurate preoperative staging to provide information about tumor location, size, configuration, and local infiltration[3]. One of the most important features of local rectal cancer staging is the assessment of the circumferential resection margin (CRM)[1] and relationship of the tumor to the mesorectal fascia (MRF), which actually defines the surgical CRM in TME surgery[4-6].

Magnetic resonance imaging (MRI) is today considered the "state-of-the-art" investigation for preoperative evaluation of pelvic malignant disease due to the method's multiplanar capabilities and its ability to visualize the rectum, the mesorectal fat and the MRF, urinary bladder and internal genitalia with high soft tissue contrast[7].

Although many studies have described the accuracy of computed tomography (CT) for predicting the depth of bowel wall and lymph node invasion[8-11], only few of them have addressed the problems of predicting tumor infiltration of the MRF with new generation of multidetector-row CT (MDCT). The current role of CT in the evaluation of patients with rectal cancer is controversial[3]. In a single examination, CT can assess the entire abdomen, pelvis and chest, allowing for local staging and distant metastases evaluation[12-15]. MRI is an integral part of the diagnostic work-up of patients with rectal cancer due to its proven efficacy to determine the tumor relationship to the MRF[4]. However, MRI does have the downside of limited availability, relatively long image acquisition time and high cost[4]. Moreover, not all patients can undergo MRI because of claustrophobia or the presence of metal in patients' bodies. In addition, another important factor in the preoperative assessment of primary rectal cancer is the frequent presence of distant disease at the time of diagnosis. Modern CT techniques are better suited than MRI to search for the local tumor extent and distant metastases in the same imaging session[16,17]. These considerations on one hand, and improved spatial resolution of new MDCT scanner on the other hand, have revived the discussion whether to use CT or MRI for rectal cancer staging[4,17]. The introduction of MDCT allowed thin-collimation scanning and high spatial resolution[1], resulting in improved multiplanar reconstructions (MPR)[3,17]. MPR images can be potentially useful for local staging in rectal cancer as they can be aligned parallel or perpendicular to the axis of the tumor similar to MR imaging. The aim of the present study was to evaluate the accuracy of high row number MDCT for the prediction of tumor invasion of the MRF being MRI findings as reference standard, and whether the addition of high-resolution MPR images can provide greater accuracy.

MATERIALS AND METHODS

Patients

One hundred and thirty-one patients with biopsy-

proven adenocarcinoma of the rectum and distal margin of the tumor within 15 cm from the anal verge were enrolled in this retrospective study.

The standard workup for patients with a rectal cancer includes a pelvic MRI for the assessment of loco-regional staging and MDCT study to determine the whole body staging.

For this reason the inclusion criteria were: (1) a biopsy proven rectal cancer (0-15 cm from anal verge according to endoluminal biopsy); (2) availability of MRI study of lower abdomen; (3) availability of contrast enhanced MDCT of the chest and abdomen examinations; and (4) both MRI and CT images performed before application of any neo-adjuvant therapy or surgery.

Exclusion criteria were: (1) previous neo-adjuvant therapy for rectal cancer; (2) contraindications to MRI examination; (3) contraindications to contrast enhanced CT imaging (*e.g.*, intolerance/allergy to iodine contrast medium); (4) insufficient MR imaging quality (*e.g.*, movement artifact) and insufficient CT imaging quality (*e.g.*, owing to metal implants); and (5) absence of one of the two diagnostic tools between MRI and CT .

Forty patients were excluded from this study: 2 patients had hip prostheses (important beam hardening artifacts reduced CT images quality); 6 patients were excluded due to movement-related artifacts in MR study; 19 patients had only MRI evaluation and 13 had a CT evaluation alone (patients in which the local MRI staging was performed in another Hospital).

A final cohort of 91 patients (65 male and 26 female, with a mean age of 69 years - range 30 to 89 years) satisfied the inclusion criteria and were enrolled in this study.

The mean interval time between the MRI and CT examination was 37 d (range 0-79 d).

The approval for this study was obtained by the ethical approval committee at our Institution.

CT imaging technique

All MDCT examinations were carried out without luminal rectal contrast media or air insufflation. All CT studies were performed on a 256-slice CT system (Brilliance iCT, Philips Medical Systems, Best, the Netherlands) with the following scan parameters: thickness 2 mm; increment 1 mm; collimation 128 × 0.625; pitch 0.915; rotation time 0.4 s; FOV 350; matrix 512 × 512. The scan images were acquired before and after the intravenous bolus injection of non-ionic iodinated contrast material (Xenetix 350; Guerbet, Aulnay, France), according to the body weight, at a rate of 3.5 mL/s, using a double-syringe injector (Medrad Stellant, Pittsburgh, PA, United States) and 18-gauge catheter positioned into the antecubital vein. Bolus tracking software was used to set individual acquisition times for the arterial, portal and equilibrium phases. Contrast material enhancement was automatically calculated by placing the region of interest cursor over the abdominal aorta, and the level of the trigger threshold was set to increase to 120 HU.

Thirteen seconds after the trigger threshold had been reached, arterial phase CT data acquisition began automatically. The portal venous and equilibrium phases were acquired after 60 and 140 s, respectively, after the trigger threshold had been reached.

Examinations were performed during one breath-hold from the thorax to the anus.

None of the patients received a contrast enema or bowel relaxation.

MRI technique

MRI imaging examination was performed for tumor staging before starting the treatment or surgery.

All MRI examinations were performed with a 1.5-T system (Achieva Plus; Philips, The Netherlands) in combination with a five-channel phased-array body coil.

After a planning scan, axial and sagittal T2 weighted turbo spin-echo (T2WI-TSE) images covering entire length of the rectum were acquired and used to plan high resolution scans.

Scan protocol consisted of axial TSE T1 weighted axial sequence turbo spin-echo (TSE) (slice thickness: 3 mm; slice: 20; gap: 3 mm; TR: 612 ms; TE: 14 ms; flip angle: 90°; FOV: 180; RFOV: 85; matrix: 272 × 320; NSA: 4; time: 4.43 min); sagittal TSE T2 sequence (slice thickness: 3 mm; slice: 32; gap: 0 mm; TR: 5501 ms; TE: 85 ms; flip angle: 90°; FOV: 220; RFOV: 105; matrix: 276 × 200; NSA: 4; time: 4.40 min); axial TSE T2 sequence (slice thickness: 3.5 mm; slice: 18; gap: 3.5 mm; TR: 4750 ms; TE: 120 ms; flip angle: 90°; FOV: 180; RFOV: 85; matrix: 256 × 256; NSA: 4; time: 3.05 min); coronal TSE T2 sequence (slice thickness: 3 mm; slice: 20; gap: 0.5 mm; TR: 5058 ms; TE: 125 ms; flip angle: 90°; FOV: 180; RFOV: 100; matrix: 256 × 256; NSA: 4; time: 3.47 min). The axial and coronal oblique images were performed orthogonal and parallel, respectively, to the long axis of the rectal cancer.

Afterwards diffusion weighted images with background body signal suppression (DWIBS) using a Multi-slice Spin Echo Eco-planar Single Shot (SE-EPI-SSh) sequence were obtained; DWIBS were combined with a short time inversion recovery (STIR) pre-pulse for fat saturation. The DWIBS sequences were acquired in a pure axial plane in order to avoid distortion artifacts, with *b*-value 0 and 1000 s/mm^2 with following parameters: slice thickness: 6 mm; slice: 12; gap: 6 mm; TR: 3000 ms; TE: 74 ms; flip angle: 90°; *b*-value: 0 and 700 s/mm^2; FOV: 380; RFOV: 80; matrix: 240 × 256; NSA: 4; time: 1.30 min; SENSE factor: 1.5. According to recent literature no contrast enhanced dynamic or steady state T1 weighted or fat suppressed

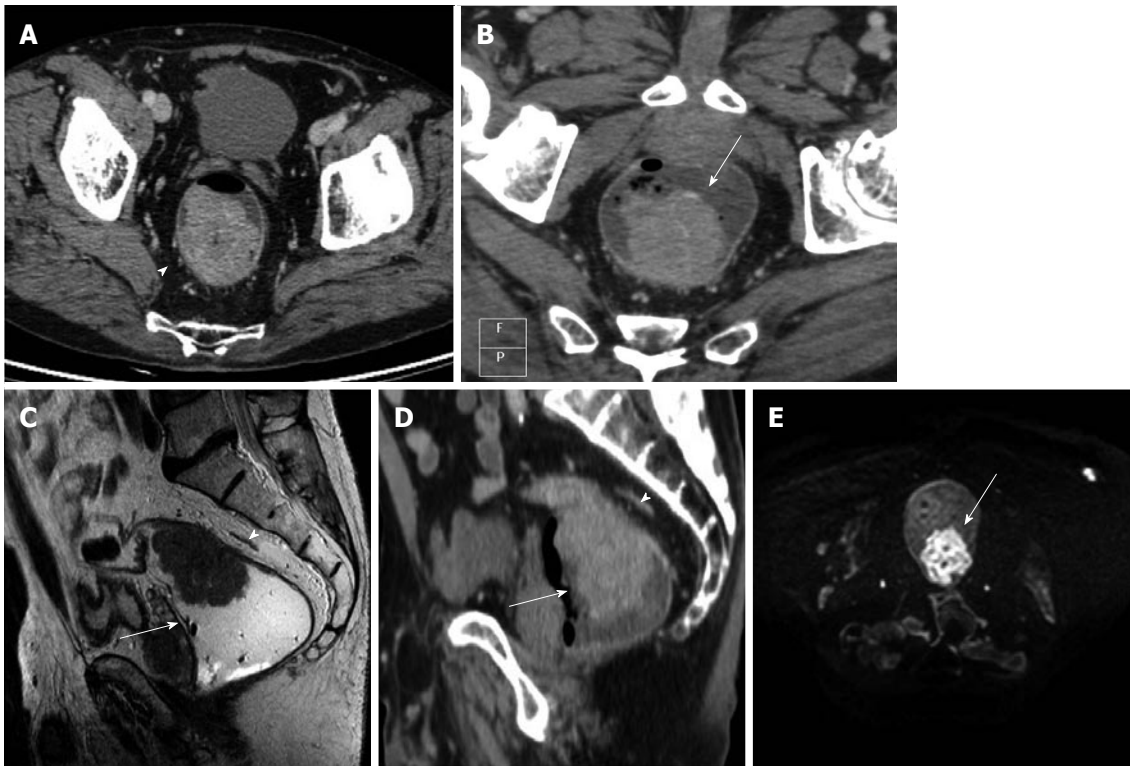

Figure 1 Images obtained in a 54 years-old man with middle-high rectal cancer. A: The pure axial contrast enhanced computed tomography (CE-CT) image shows a tumor, as a intraluminal polypoid mass, with spiculated configuration margin and spread through the mesorectal fat. The tumour does not involve the mesorectal fascia (MRF) (arrowhead); B: Multiplanar reconstruction (MPR) MDCT images, along the axial plane of tumour axis, shows the presence of the tumor (arrow) with no involvement of the MRF; C: T2 (TSE) MRI image of the same patient (sagittal plane), shows the tumor as a polypoid mass (arrow), along the posterior burden of the rectum infiltrating through the muscularis propria into the mesorectal fat without MRF involvement (arrowhead); D: CE-CT MPR image (sagittal slice), at the same level shows the tumour as a polypoid mass inside the rectal lumen infiltrating the mesorectal fat without MRF involvement (arrowhead); E: DWIBS image (*b*-value 1000), the tumor presents high signal, due to restricted water diffusion; the normal rectal wall or the surrounding tissues have a lower signal intensity in comparison with the polypoid mass. The mesorectal (arrow) fascia is not detectable.

sequences were used[18,19].

All these sequences were obtained in free breathing. The total examination time was approximately 30 min. Patients did not undergo any preparation such as bowel cleaning or spasmolytic medication before the MR examinations. Luminal distention was achieved with rectal administration of a small amount (almost 100 mL) of sonography transmission gel to distend the rectal lumen.

CT image analysis

In order to obtain an optimal contrast enhancement, the images of the pelvis were observed in the portal-venous contrast enhanced phase. Multiplanar CT reconstructions were performed from the same radiologist, (blinded to pathological evaluation, clinical and MRI patient data), that analyzed all the CT images and orientated MPR images axial plane along the tumor axis.

According to recent guidelines about clinical management of rectal cancer patients with MRI (recommendations from ESGAR, 2012)[18], sagittal reconstructions are used to determine the longitudinal tumor axis in order to angle the axial and coronal planes as perpendicular and parallel to the tumor axis as possible, respectively. MPR CT images were performed following the same recommendations to obtain axial and oblique coronal planes similar to MRI imaging.

After iv contrast injection the tumor was seen as an intraluminal polypoid mass (Figure 1) or as asymmetric or circumferential mural thickening (> 6 mm)[20] with or without luminal narrowing (with abrupt transition from normal to abnormally thick-walled rectum) and smooth outer bowel margins. In some cases strands of the soft tissue extending from serosal surface into perirectal fat was observed (Figure 1).

The MRF was seen as a thin, curvilinear structure surrounding the mesorectal fat with similar density to muscle adjacent to the rectum[1,21] (Figures 2 and 3). The main outcome parameter was the involvement of MRF defined as a visible fat line between the tumor and the MRF (Figure 3).

All CT axial images were observed first, in order to determine the tumor extension and direct involvement of the MRF; few days after (0-6 d), the same evaluation was done with MPR images.

MRI analysis

For each patient a radiologist with 10 years of experience in abdominal imaging analyzed T2-weighted

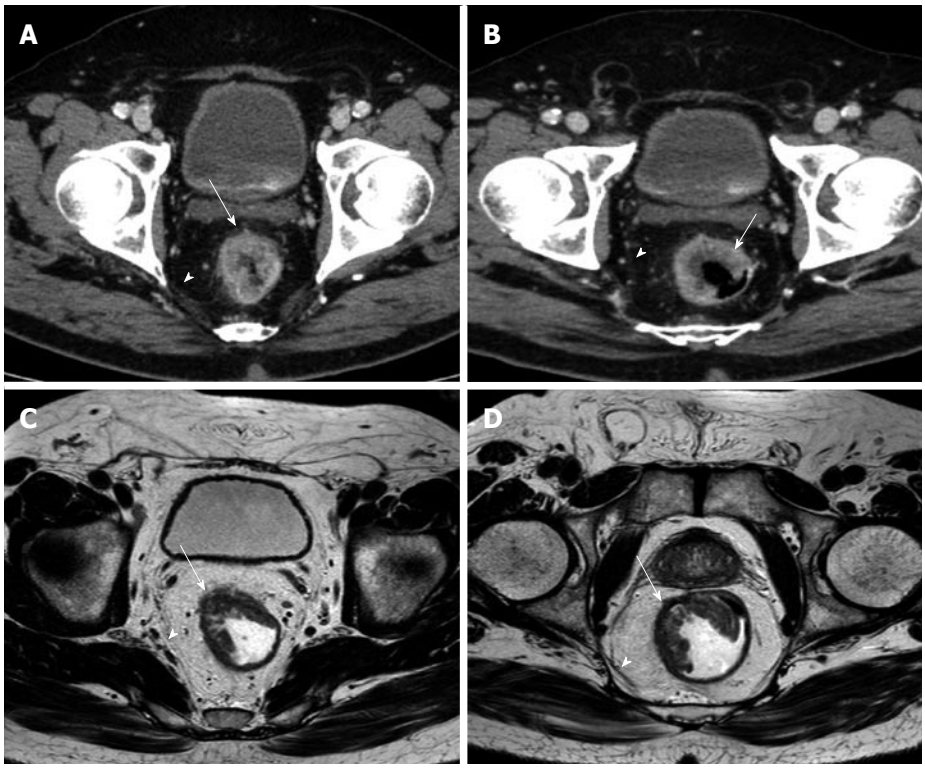

Figure 2 Images obtained in a 64 years-old man with middle rectal cancer. A: Axial contrast enhanced computed tomography (CE-CT) image shows the tumour as an irregular mural thickening of the anterior rectal wall with possible infiltration into the perirectal fat. The mesorectal fascia is seen as a thin line (arrowhead) surrounding the mesorectal fat and is not involved by the tumor (arrow); B: Multiplanar reconstruction (MPR) para-axial CE CT image of the same patient shows the tumour as an irregular mural thickening of the anterior rectal wall (arrow) with no infiltration into the perirectal fat. The mesorectal fascia (arrowhead) is better defined in MPR para-axial CT image; C: T2 (TSE) MRI image of the same patient shows the tumour as a lesion of the anterior rectal wall, slightly hyperintense compared to the muscle, that extends through the hypo-intense muscle layer into the perirectal fat (arrow) and without mesorectal fascia involvement (arrowhead); D: Orthogonal axial high-resolution T2-weighted MR image of the same patient shows an intraluminal mass (arrows) confined to the intact, hypo-intense muscularis propria (the proper muscle layer is shown as a low intensity band (*). The mesorectal fascia (arrowhead).

sequences and DWIBS images in order to detect and correctly localize the primary lesion. The presence of the tumor was diagnosed on T2-weighted sequences. Rectal cancer typically appeared hypointense as compared to the surrounding fat, and slightly hyperintense as compared to the muscles.

The mesorectal fascia was seen as a thin hypo-intense line surrounding the mesorectal fat[1].

DWIBS images were analyzed in order to obtain information about microscopic structures of biologic tissue through water proton mobility and to achieve a possible tool to monitor the response of tumor tissue after therapy[22].

These images were of diagnostic quality and adequate to identify the tumor region. When the anatomic details were unclear due to the low signal-to-noise (SNR) on DWIBS images, they were matched to T2WI images of same planes. The diagnostic criterion on DWI was defined as a focal mass with high signal intensity (SI) on b1000 DW, compared with the signal of the normal adjacent rectal wall or background of lower SI tissue[22].

During images analysis, the radiologist was blinded to clinical patient data and pathological evaluation.

Multiplanar T2 weighted sequences and axial T1 weighted sequences images were evaluated in order to assess the presence of the tumor, the involvement of the MRF and the adjacent structures.

Statistical analysis

All statistical analysis was performed using commercially available software (Med Calc, Med calc software 11.0, Mariakerke Belgium). The McNemar test was used to compare axial and MPR CT images with those of MRI imaging, which was considered as the reference standard, in order to determine the involvement of the MRF.

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of axial and MPR images were assessed and the obtained data were then compared. Overall accuracy, sensitivity and specificity of the prediction of involvement of the MRF were calculated using cross-tabulation statistics.

RESULTS

In the native axial CT imaging analysis the MRF was involved by the tumor in 51 patients, while on MPR images the involvement of the MRF was observed in 50 patients. At MR image evaluation, the involvement of the MRF by the rectal cancer was observed in 51 patients.

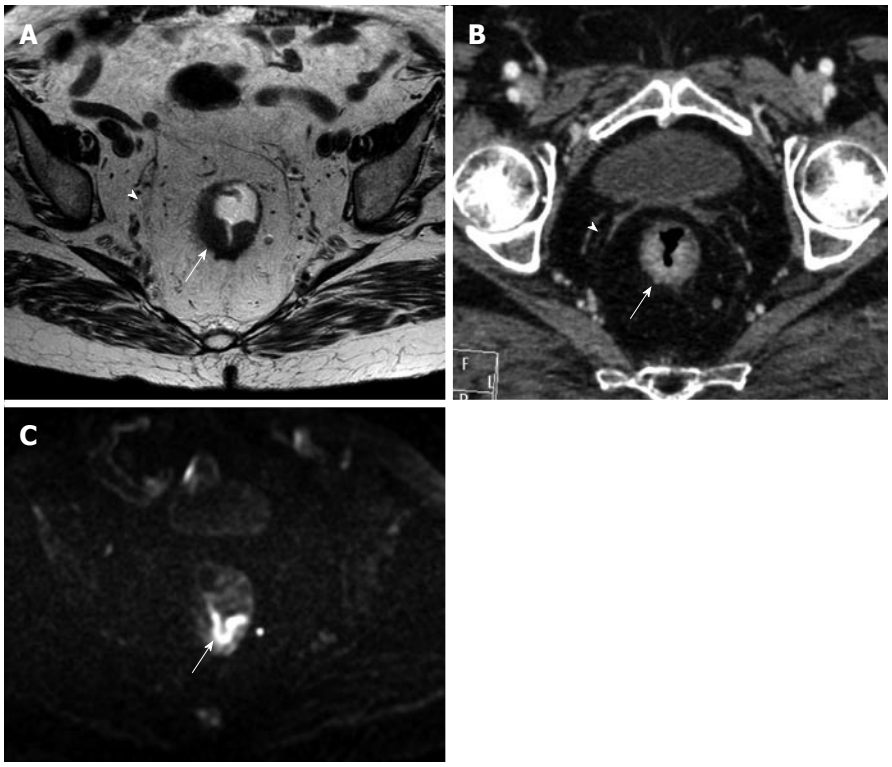

Figure 3 Images obtained in an 86 year-old woman with middle rectal cancer. A: Orthogonal axial high-resolution T2-weighted MR image shows the tumor as a thickening (arrow) along the posterior aspect of the rectum, infiltrating through the muscularis propria (the band of proper muscle layer (*) is destroyed) into the mesorectal fat. The mesorectal fascia is seen as a thin hypointense line (arrowhead) surrounding the mesorectal fat; B: Multiplanar reconstruction (MPR) para-axial contrast enhanced computed tomography image of the same patient shows the tumour as an irregular mural thickening of the posterior rectal wall with spiculations extending into the peri-rectal fat. The mesorectal fascia is well defined and not involved (arrowhead); C: DWIBS image (*b*-value 1000), the tumor (arrow) is clearly recognizable as high signal in comparison with the lower signal intensity of the normal rectal wall.

Table 1 Summarizing table of number of patient correctly staged with multiplanar reconstruction and axial computed tomography images in comparison of magnetic resonance imaging

Image analysis	TN[1]	FN[2]	TP[3]	FP[4]	TOT[5]
CT-axial	30	10	41	10	91
CT-MPR	35	6	45	5	91
MRI	40		51		91

[1]TN: True negative. Number of patients in which the MRF was correctly considered not involved with axial and MPR imaging according to MRI; [2]FN: False negative. Number of patients in which the MRF was wrongly not considered involved with axial and MPR imaging, but it was with MRI; [3]TP: True positive. Number of patients in which the MRF was correctly considered involved with axial and MPR imaging according to MRI; [4]FP: False positive. Number of patients in which the MRF was wrongly considered involved with axial and MPR imaging, but it wasn't with MRI; [5]TOT: Total of patients. CT: Computed tomography; MPR: Multiplanar reconstruction; MRI: Magnetic resonance imaging.

DWIBS allowed to recognize the tumor as a focal mass with high signal intensity on high b-value images, compared with the signal of the normal adjacent rectal wall or with the lower tissue signal intensity background (Figures 1, 3 and 4).

The overall correlation of MPR and native axial findings with the MR images demonstrated (Table 1) that the number of patients correctly staged by evaluating the native axial images was 71 out of 91 patients (41 true positive, TP; 30 true negative, TN), while by using the MPR a total of 80 patients were correctly staged (45 TP and 35 TN) (Figure 4).

The number of false negative (FN) for axial and MPR was respectively 10 FN and 6 FN. The results obtained in our series of patients show an overall good diagnostic value of CT technique: considering the native axial CT images, the overall sensitivity and specificity were respectively 80.4% and 75%, PPV was 80.4%, NPV 75% and Accuracy was 78%. While analyzing the MPR images the sensitivity raised up to 88% and specificity up to 87.5%, PPV was 90%, NPV 85.36% and accuracy raise up to 88% (Table 2). The difference in performance between axial and MPR images was not statistically significant, in terms of sensitivity and specificity (McNemar test with $P = 0.22$ and $P = 0.13$ respectively) (Table 3), but considering the overall diagnostic accuracy, in terms of MRF involvement, the MPR images demonstrated to be superior ($P = 0.02$) in comparison with native axial images alone, as compared to the reference MR images (Table 4).

DISCUSSION

To date, only few studies[1,4,16,17,21,23,24] analyzed the role

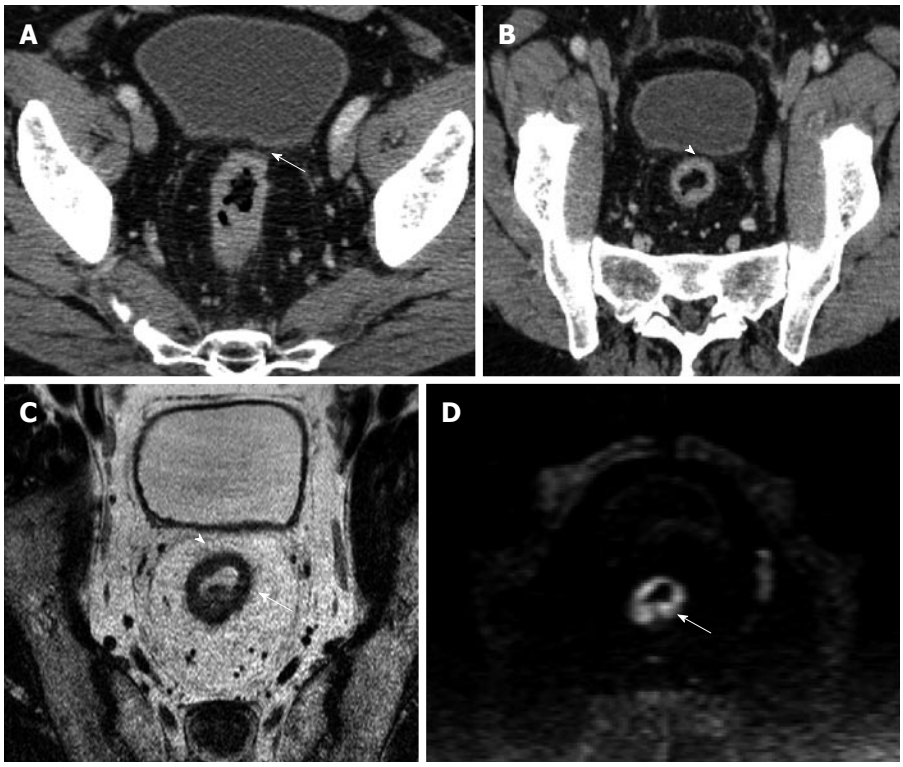

Figure 4 Images obtained in a 68 years-old man with high rectal cancer. A: Axial computed tomography (CT) image shows a tumor as a circumferential thickening in the bowel wall; in the anterior wall (arrow) the tumor seems to involve the mesorectal fascia (MRF); B: Multiplanar reconstruction CT images shows a visible fat line between the tumor and the MRF (arrowhead); C: Axial T2-weighted magnetic resonance image shows a rectal wall involvement by the tumor but also a wide fat pad between the tumor and the free MRF; D: DWIBS image (*b*-value 1000), the tumor is depicted as a high signal circumferential thickening of the bowel wall, in comparison with the lower signal intensity of surrounding tissue.

Table 2 Summarizing table of sensitivity, specificity, positive predictive value, negative predicting value and accuracy of axial and multiplanar reconstruction computed tomography images in correctly identify the involvement of the mesorectal fascia in comparison of magnetic resonance imaging

Axial CT images				
Sensitivity	Specificity	PPV	NPV	Accuracy
80.40%	75%	80.4%	75%	78%
MPR CT images				
Sensitivity	Specificity	PPV	NPV	Accuracy
88%	87.5%	90%	85.36%	88%

CT: Computed tomography; PPV: Positive predictive value; NPV: Negative predicting value; MRF: Mesorectal fascia; MRI: Magnetic resonance imaging.

Table 3 Summarizing table of McNemar test calculation to determine the statistical significant of sensitivity and specificity between axial and multiplanar reconstruction computed tomography images in comparison to magnetic resonance imaging

	Axial- subjects	Axial+ subjects	Total of subjects with MRI+
Sensitivity[1] (P = 0.22)			
MPR- subjects	5	1	6
MPR+ subjects	5	40	45
Total subjects of MRI+	10	41	51[n]
	AXIAL- subjects	AXIAL+ subjects	Total of subjects with MRI-
Specificity[2] (P = 0.13)			
MPR- subjects	29	6	35
MPR+ subject	1	4	5
Total of subjects with MRI-	30	10	40[n]

McNemar test: 2 × 2 contingency table, which tabulates the outcomes of the two tests (axia CT and MPR images) on a sample of n subjects (respectively 51 who were positive with MRI and 40 who were negative). [1]P = 0.22, two tails, not statistically significant; [2]P = 0.13, two tails, not statistically significant. CT: Computed tomography; MRI: Magnetic resonance imaging.

of MDCT as possible and reliable imaging technique in assessment of MRF invasion by rectal cancer. According to recent literature[25], the appropriate angulation of the axial plane orthogonal to the tumor is essential in primary tumor staging, since incorrect plane obliquity leads to a pseudospiculated appearance that may lead to overstaging (Figures 2 and 4). Placement of the orthogonal plane is based on the definition of the tumor on sagittal T2-weighted images.

DWIBS are usually performed in pre-operative rectal cancer staging[22,25] in order to improve the detection and localization of rectal tumors, especially when the tumor is difficult to visualize with other sequences[25]. While these sequences have no role in the assessment of mesorectal fascia involvement, due to the intrinsic limitations of MRF visualization

Table 4 Summarizing table of McNemar test calculation to determine the statistical significant of accuracy between axial and multiplanar reconstruction computed tomography images in comparison to magnetic resonance imaging

	Uncorrect subjects staged with AX/MPR CT images	Correct subjects staged with AX/MPR CT images	Total of correct subjects staged with MRI
Accuracy[1] ($P = 0.02$)			
Uncorrect subjects staged with MPR/AX	9	2	11
Correct subjects staged with MPR/AX	11	69	80
Total of correct subjects staged with MRI	20	71	91

McNemar test: 2 × 2 contingency table, which tabulates the outcomes of the two tests (axial CT and MPR images) on a sample of n subjects (91 correct staged with MRI). [1]$P = 0.02$, two tails, statistically significant. MPR: Multiplanar reconstruction; MRI: Magnetic resonance imaging; CT: Computed tomography.

at high b-values. While the DWIBS are frequently employed in restaging of rectal cancer patients, due to the possibilities to offer information about structures of biologic tissue through water proton mobility, and suggested as a possible tool to monitor the response of tumor tissue after therapy[22,25].

Several problems frequently arise during this critical initial step, due to motion artifacts, small tumor size, low contrast between the tumor and the rectal wall on fast relaxation fast spin-echo (FSE) T2-weighted images, redundancy and tortuosity of the rectum. In addition, nodes along the pelvic sidewall and superior rectal vessels may fall outside the FOV of axial high-resolution images.

In this setting the clinical use of MDCT images combined with MPR along the different axis of rectal lumen, permits to overcoming some of these limitations, having also the possibility to include a large FOV and modify the different perpendicular axial plane in a less time consuming analysis in order to evaluate the tumor axis (also in case of tortuosity and redundancy of the rectum), the MRF involvement and distant lymph-nodes sites. Moreover the new generation multidetector row CT scanner permits to increase the spatial resolution, offering high detailed images combined with short acquisition time and avoiding or reducing possible motion artifacts. Unfortunately, for small size rectal tumor, there's a lower contrast between the tumor and the rectal wall using CT images compared to MR images, especially if combined with use of DWIBS.

A recent survey of United Kingdom practice has revealed that less than 50% of patients were offered MR staging and up to 80% of patients who do not undergo MR staging have a CT examination[1]. The results obtained in this study may help to establish MDCT as an effective diagnostic technique in the evaluation of preoperative local staging of rectal cancer[3].

In our study we compared the diagnostic capability of MDCT images, with new generation of multi-row scanner, in the prediction of MRF involvement by rectal cancer, by evaluating native axial images and MPRs, as compared with MR images as reference standard[1,4,16,23]. In our series of patients a good diagnostic quality was achieved for both series of CT images, obtaining an accuracy of 78% for pure axial images and 88% for MPR (difference statistically significant, $P = 0.02$), while the sensitivity of pure axial images was 80.4% and these results arise to 88% with MPR. Previous studies reported high accuracy rates for CT[3,17,21], however, most patients in these early series had advanced disease[3,26]. In more recent reports, a less satisfactory results have been obtained, with accuracy rates ranging between 41% and 82%[3,4,16,24] in rectal cancer. Those results, probably, were related to the limited spatial collimation and insufficient reconstructions increments used in CT-protocol (*i.e.*, thickening from 5 to 10 mm, no MPRs)[4,16,25] as well as the absence of standardized contrast agent injection protocol. Therefore, the spatial resolution of the scans was too low to make any reliable predictions on margin involvement, especially if compare with MR protocol (assumed with 3 mm thickness and with different orientation of the axial plane)[16,23,27]. In comparison with previous studies we obtained an higher PPV (90%); this result could be explained by the use of thinner slices (2 mm), increasing consequently the spatial resolution, close to MR images protocol (3 mm). The employed protocol, 2 mm thickness and 1 mm of increment, offers reliable results comparable with MR images, especially with the use of MPR.

Our findings are more similar to those of Shina[1] that found an accuracy rate in predicting the involvement of mesorectal fascia in comparison with histopathology of 96.5% and 91.2% on MPR and axial images, respectively. Multiplanar reconstructions images in addiction to axial images significantly improve the diagnostic accuracy in image interpretation ($P = 0.02$), even if the difference of sensitivity and specificity between axial images and MPR not reach the statistical significant ($P > 0.05$). In the study of Matsuoka[28] the accuracy of MDCT (4 slices, 5 mm thickness) and MRI was assessed using the histopatology as gold standard, with equal results between CT and MRI in the preoperative local staging of rectal carcinoma. In our series of patients the NPV of MDCT was 75% for axial images and 85.36% for MPRs, with specificity of 75% and 87.5% respectively.

One of the limitations of this study is represented by the use of MRI as reference standard, rather than histology, although this comparison is virtually impossible since patients with a MRF involvement are currently treated with long courses of chemoradiation therapy[4,29]. In addiction we did not perform any luminal distention on CT images and this could

explaining some discrepancies of finally rectal cancer findings, between CT and MRI analysis. Another limitation of CT images is represented by the fact that in patients with small amounts of peri-rectal fat, the identification of the true extramural extension is more challenging due to smaller tissue interfaces, causing a higher rate of mistakes in assessment of involvement of the MRF. In our series, we did not considered the BMI of the patient as well as the amount of peri-rectal fat, in order to obtain a reliable data about sensitivity of CT images, in daily current clinical practice.

Moreover, as well known, CT-images do not allow accurate differentiation of different bowel layers, as compared with MRI, but the involvement of the MRF represents the main aim of rectal cancer imaging, since the MRF involvement determines the distinction between primary resectable and locally advanced tumors[1].

In conclusion, despite these limitations the CT imaging of rectal cancer patients with new generation MDCT scanner, demonstrated high sensitivity and high accuracy in assessment of MRF involvement, especially with the use of MPRs, and would become a potential one-step imaging tool. CT imaging could be useful as making decision therapy process during a whole-body staging workup, allowing accurate distant rectal staging and local involvement of the MRF in a single examination.

COMMENTS

Background

Treatment options in rectal cancer are total mesorectal excision (TME) or preoperative neoadjuvant radiochemotherapy in patients with locally advanced rectal cancer (LARC). One of the most important features of local rectal cancer staging is the assessment of the tumor relationship with the mesorectal fascia (MRF), which defines the circumferential resection margin (CRM) in TME surgery. To date MR imaging investigation is used for local staging and to identifying patients who may benefit from preoperative chemotherapy-radiation therapy (patients in which the MRF and the CRM could be involved by the tumor). However not all patients can undergo MRI because of claustrophobia or the presence of metal in patients' bodies; moreover MRI has the downside of limited availability, relatively long image acquisition time and high cost. Another important factor in the preoperative assessment of primary rectal cancer is the frequent presence of distant disease at the time of diagnosis, which are assessed, routinely, with CT. For these reasons, the use of MDCT for local staging and distant metastases evaluation could offer high detailed images combined with low cost and short acquisition time.

Research frontiers

New generation of high row number MDCT scans allow thin-collimation, high spatial resolution and better multiplanar reconstructions (MPRs). MPR images can be aligned parallel or perpendicular to the axis of the tumor similar to MR imaging and can be useful for predicting tumor infiltration of the MRF in local staging of rectal cancer. Therefore MDCT can assess in a single examination, the entire abdomen, pelvis and chest, allowing for local staging and distant metastases evaluation.

Innovations and breakthroughs

Considering the variability among the results in previous studies, the actual evidence suggests that old CT protocol, having a limited spatial collimation, an insufficient reconstructions increments and poor MPRs, could not be used for local staging in rectal cancer. New generation MDCT scanner used in modern clinical practice, with high sensitivity and high accuracy in assessment of MRF involvement, would become a potential one-step imaging tool for distant rectal cancer staging and local involvement of the MRF.

Applications

The importance of this work relies on the possibility to offer, in a single step examination, a new diagnostic approach (performed with new generation MDCT,) that allows the non-invasive evaluation of MRF involvement in local rectal staging, as well as the assessment of distant metastases using high detailed images of the entire abdomen, pelvis and chest. Moreover in this manuscript the authors compared and commented our results with those of previous literature on this field by using the two different techniques modalities (*i.e.*, CT and MRI).

Terminology

TME is a surgical technique that entails en bloc resection of the primary tumor and the mesorectum by means of dissection along the mesorectal fascial plane or the CRM. MDCT are new generation of CT with high number of detector, which allow to obtain high spatial resolution images with thinner collimation. MPR is multiplanar reconstructions of the images are images obtained after a post-processing of native axial CT images. Thanks to high collimation of MDCT, all pure axial images can be orientated along different planes (*i.e.*, coronal, sagittal, and oblique axis). MRF is mesorectal fascia, surrounds the mesorectal fat around the rectum. The mesorectal fascia runs along the anterior aspect of the sacrum, where it fuses with the presacral fascia, and then laterally on either side of the rectum. Anteriorly in males, it forms a dense band of connective tissue posterior to the seminal vesicle and prostate gland (the Denonvilliers fascia). The MRF is critical for surgical planning in TME. On T2-weighted images appears as a thin hypointense line surrounding the meserectal fat. On CT images is depicted as a thin line surrounding the mesorectal fat with similar density to the muscles.

Peer-review

Congratulations for the article. Often in daily clinic are situations where you can not perform an MRI either clinical or resource problems. Having information like that concludes this article endorse the decisions of physicians to such situations and allow proper staging of patients.

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ORIGINAL ARTICLE

Retrospective Study

Clinical and *ABCB11* profiles in Korean infants with progressive familial intrahepatic cholestasis

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Author contributions: Ko JS and Moon JS designed the study; Park SS performed the genetic analyses; Park JS and Seo JK collected and analyzed the clinical data and wrote the paper.

Institutional review board statement: This study was carried out after obtaining the clearance from the ethical board of the hospital (GNUH 2015-09-004-001).

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Abstract

AIM: To investigate clinical profiles and mutations of *ABCB11* in Koreans with progressive familial intrahepatic cholestasis 2 and review the differences between Koreans and others.

METHODS: Of 47 patients with neonatal cholestasis, five infants had chronic intrahepatic cholestasis with normal γ-glutamyl transpeptidase. Direct sequencing analyses of *ABCB11*, including exons and introns, were performed from peripheral blood.

RESULTS: Living donor-liver transplantation was performed in four patients because of rapidly progressive hepatic failure and hepatocellular carcinoma. Three missense mutations were found in two patients: compound heterozygous 677C>T (S226L)/3007G>A (G1003R) and heterozygous 2296G>A (G766R). The mutations were located near and in the transmembranous space.

CONCLUSION: Alterations in the transmembrane of the bile salt export pump in the Korean infants were different from those previously reported in Chinese, Japanese, Taiwanese, and European patients.

Key words: Hepatocellular carcinoma; Progressive familial intrahepatic cholestasis; *ABCB11*; Bile salt export pump

Core tip: Reports of progressive familial intrahepatic cholestasis (PFIC) mutations in Asian countries have been less than those in Western countries because of time consuming and expensive diagnostic tools. Recently, reports on mutations of *ABCB11* in Asian patients with PFIC have been increasing. In this study, the authors report mutations of *ABCB11* in Korean infants with PFIC2 and compare Korean mutations with previously reported mutations.

Park JS, Ko JS, Seo JK, Moon JS, Park SS. Clinical and *ABCB11* profiles in Korean infants with progressive familial intrahepatic cholestasis. *World J Gastroenterol* 2016; 22(20): 4901-4907 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i20/4901.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i20.4901

INTRODUCTION

Progressive familial intrahepatic cholestasis (PFIC) is an autosomal recessive disorder that manifests as cholestasis during the neonatal period due to defective bile secretion. PFIC is divided into types 1, 2, and 3 according to their different clinical manifestations and genetics. In PFIC1 and PFIC2, cholestasis develops during the neonatal period, and γ-glutamyl transpeptidase (GGT) is within normal limits. PFIC3 develops later than PFIC1 and PFIC2, and features a positive prenatal history of maternal cholestasis. Generally, cholestasis with elevated GGT is associated with PFIC3 rather than PFIC1 and PFIC2. Persistent or repetitive cholestasis develops within 1 year of age and rapidly progresses to liver cirrhosis and hepatic failure in patients with PFIC. Mutations of biliary transporters associated with PFIC have been discovered, which can aid in understanding the diagnosis and pathogenesis. The genes are *ATP8B1, ABCB11,* and *ABCB4,* which encode familial intrahepatic cholestasis 1 protein (FIC1), bile salt export pump (BSEP), and multidrug resistance protein 3 (MDR3) in PFIC1, 2, and 3, respectively.

ABCB11 is located on chromosome 2q24. It encodes BSEP, which plays a role in the secretion of conjugated bile acids, including taurocholates. BSEP defect can cause cholestasis with a normal range of GGT because of a bile secretion defect[1]. Over 82 different *ABCB11* mutations have been reported[2,3]. Of them, E297G and D482G account for 30% of BSEP mutations in European patients with PFIC2. In Asia, mutations of BSEP in Chinese, Japanese, and Taiwanese patients with PFIC2 were reported[4-7].

To the best of our knowledge, there have been fewer reports of *ABCB11* (BSEP) mutations in Asians with PFIC2 than in Europeans[2,4-7]. Because PFIC2 features rapid progression to liver cirrhosis and hepatic failure within the first decade, rapid diagnosis, management, and prediction of prognosis are important. In the present study, the authors investigated clinical profiles of Korean infants with PFIC and performed a mutation analysis on the *ABCB11* gene. The authors obtained clearance from the ethical board of the hospital (GNUH 2015-09-004-001).

MATERIALS AND METHODS

Patients

Between 2005 and 2006, 47 patients visited the Department of Pediatrics in Seoul National University Children's Hospital for neonatal cholestasis. Examinations included abdominal ultrasonography, duodenal intubation, a hepatobiliary scan, and liver biopsy. Inborn error of metabolism, total parenteral nutrition, drug related cholestasis, congenital infection, and cholestasis secondary to sepsis were excluded. PFIC was suspected based on intrahepatic cholestasis with normal ranged GGT or on the results of the genetic analyses. None had a family history of PFIC.

Genetic analyses

Genetic analyses were performed for diagnosis with parental consent. Direct sequencing analysis of *ABCB11* was done using peripheral blood. Exons and flanking intron sequences of the *ABCB11* gene (NC_000002.10) were amplified by polymerase chain reaction (PCR) from total genomic DNA. PCR products were purified by ExoSAP-IT (USB, Cleveland, OH, United States) and subjected to DNA sequencing using the BigDye v3.1 Terminator Chemistry (PE Applied Biosystems, Foster City, CA, United States), followed by separation on an ABI 3100 DNA sequencer (PE Applied Biosystems). Sequence data were analyzed manually and were assembled with the Seqscape v2.5 (PE Applied Biosystems). As reference control, the *ABCB11* genomic sequence was obtained from http://pharmacogenetics.ucsf.edu/set1/BSEPrefseq.html.

RESULTS

Among the 47 patients with cholestatic jaundice presented during the 2-year period, extrahepatic biliary atresia was diagnosed in eleven, congenital infection with TORCH in four, neonatal intrahepatic cholestasis caused by citrin deficiency in three, arthrogryposis, renal dysfunction, cholestasis (ARC) syndrome in two, neonatal Dubin-Johnson syndrome in two, Alagille syndrome in one, and non-syndromic bile duct paucity in one[8]. PFIC was suspected in five patients with intrahepatic cholestasis and normal GGT. Table 1 summarizes the clinical and laboratory findings of the patients. The chief complaint was cholestatic jaundice in all patients, and onset of the symptom ranged from 20 d to 9 mo after birth. Gallstone was developed in patient 1 and 2, and hepatocellular carcinoma (HCC) was developed in patient 1 (Figure 1). Hepatic pathologic examinations were performed

Table 1 Clinical, laboratory findings and mutations of five infants with progressive familial intrahepatic cholestasis

Patient	Sex	Age of symptom onset	Age at LTx	Associated sign	AST/ALT (IU/L) (0-37/0-41)	T/D. bil (mg/dL) (0-1.2/0-0.5)	GGT (IU/L) (6-71)	AFP (ng/mL) (0-7.0)	Mutations of *ABCB11*
1	F	20 d	24 mo	Gallstone, HCC	602/242	11.3/7.9	29	3070	S226L/ G1003R
2	F	9 mo	10 yr	Gallstone	178/242	8.2/5.2	25	< 5	G776R
3	M	5 d	6 mo	-	416/93	35.1/14	25	21500	No
4	M	1 mo	3.5 mo	-	1467/250	44.5/25.1	50	-	No
5	M	2 mo	-	-	951/677	14.3/8.0	52	770000	No

LTx: Liver transplantation; T/D.bil: Total bilirubin/direct bilirubin; GGT: γ-glutamyl transpeptidase (normal values in brackets; Laboratory values were obtained at initial visits of five cases).

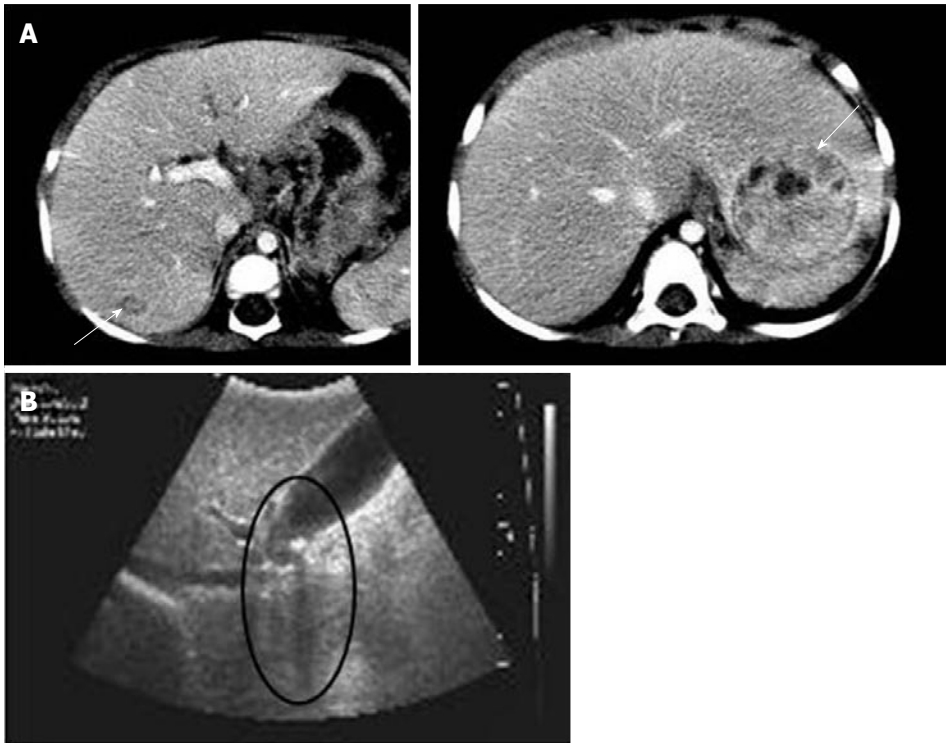

Figure 1 Radiologic hepatic evaluations in patient 1 and 2. A: Abdominal computed tomography of patient 1 revealed two contrast-enhanced hepatic masses (arrows) at 21 mo of age; B: Gallstone and its posterior shadow (circle) were observed on liver ultrasonography in patient 2.

in all patients. Various degrees of periportal fibrosis and inflammatory cell infiltration, canalicular and cytoplasmic bile pigments, cholestasis, and bile ductular dilatation and proliferation were noted in all hepatic specimens of the patients. HCC was confirmed from an excised liver in patient 1 (Figure 2). Living donor liver transplantations were performed in four patients due to hepatic failure between 2.5 mo and 10 years after their initial visits. Three of the 10 alleles examined showed mutations: compound heterozygous 677C>T (S226L)/3007G>T (G1003R) in patient 1 and heterozygous 2296G>A (G776R) in patient 2 (Figure 3). Three of the five patients showed no mutation of *ABCB11*. The PolyPhen program (http://genetics.bwh.harvard.edu/pph2) predicted that S226L is probably benign with a score of 0.175, but the SIFT program (http://sift.jcvi.org) predicted that S226L deleteriously affects protein function. G1003R and G776R were predicted to be probably damaging with scores of 1.000 by the PolyPhen-2 program and to affect deleteriously protein functions. 3007G/T (G1003R) was a novel mutation of *ABCB11*.

DISCUSSION

Three mutations were found in two patients with the PFIC phenotype: 677C>T in exon 8 (S226L) and 3007G>A in exon 23 (G1003R) in patient 1 and 2296G>A in exon 19 (G766R) in patient 2 (Figure 3). Patients with mutations presented with chronic intrahepatic cholestasis with normal GGT from the infantile period, rapidly declining liver functions, gallstone without intravascular hemolysis, and HCC (Table 1, Figures 1-3).

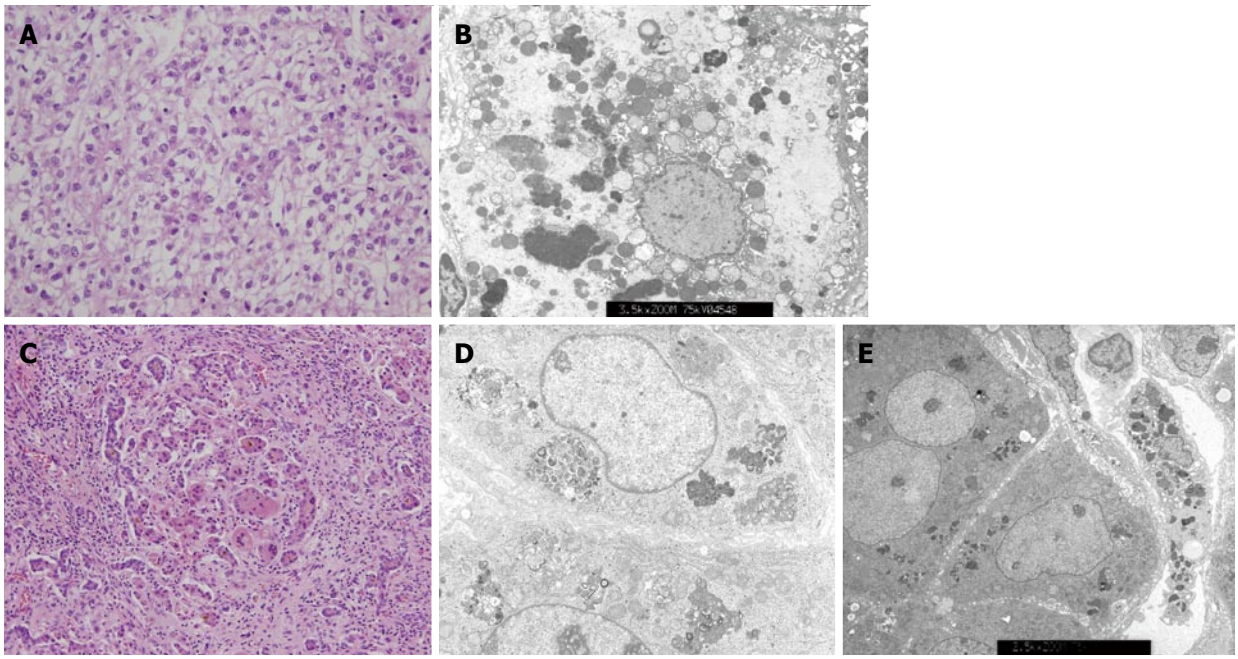

Figure 2 Liver histologic features from infants with chronic intrahepatic cholestasis with normal ranges of γ-glutamyl transpeptidase. A: Hepatocellular carcinoma was confirmed by liver specimen at hepatectomy taken from patient 1 at 24 mo of age. Cellular atypia with trabecular and acinar type was shown. Microvascular invasion was not identified; hematoxylin-eosin stain, original magnification × 400; B: Electron microscopic examination of liver specimen from patient 2 shows many globular or curly appearance electron dense materials in the cytoplasm with original magnification of × 3.5k; C: Liver biopsy at hepatectomy taken at 6 months of age from patient 3 shows periportal fibrosis, inflammatory cell infiltration, intracanalicular bile plugs, giant cell formation, and bile ductular proliferation; hematoxylin-eosin stain, original magnification × 200; D: Electron microscopic examination of liver specimen from patient 4 at 3.5 mo of age reveals amorphous and coarse granular bile pigments in the dilated bile canaliculi. Original magnification × 5.0k; E: Electron microscopic examination of liver specimen from patient 5 shows aggregated bile pigments in the cytoplasm. Original magnification × 2.5k.

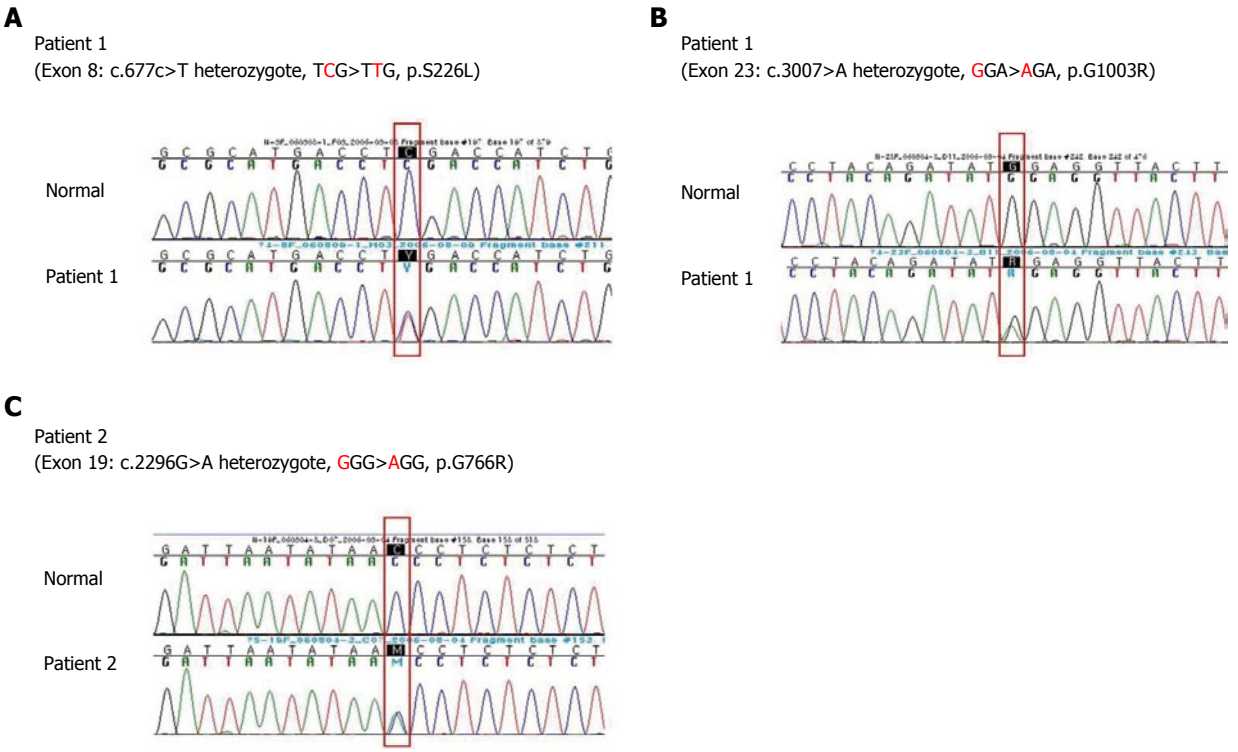

Figure 3 Direct sequencing analysis of the *ABCB11* genes demonstrating (A) heterozygous C to T substitution in exon 8 predicting a missense mutation at amino acid position 226(p.S226L) (B) heterozygous G to A in exon 23 predicting a missense mutation at amino acid position 1003 (p.G1003R), and (C) heterozygous G to A in exon 19 predicting a missense mutation at amino acid position 776(p.G776R), (A) and (B) were detected in *ABCB11* gene of patient 1 and (C) was detected in patient 2.

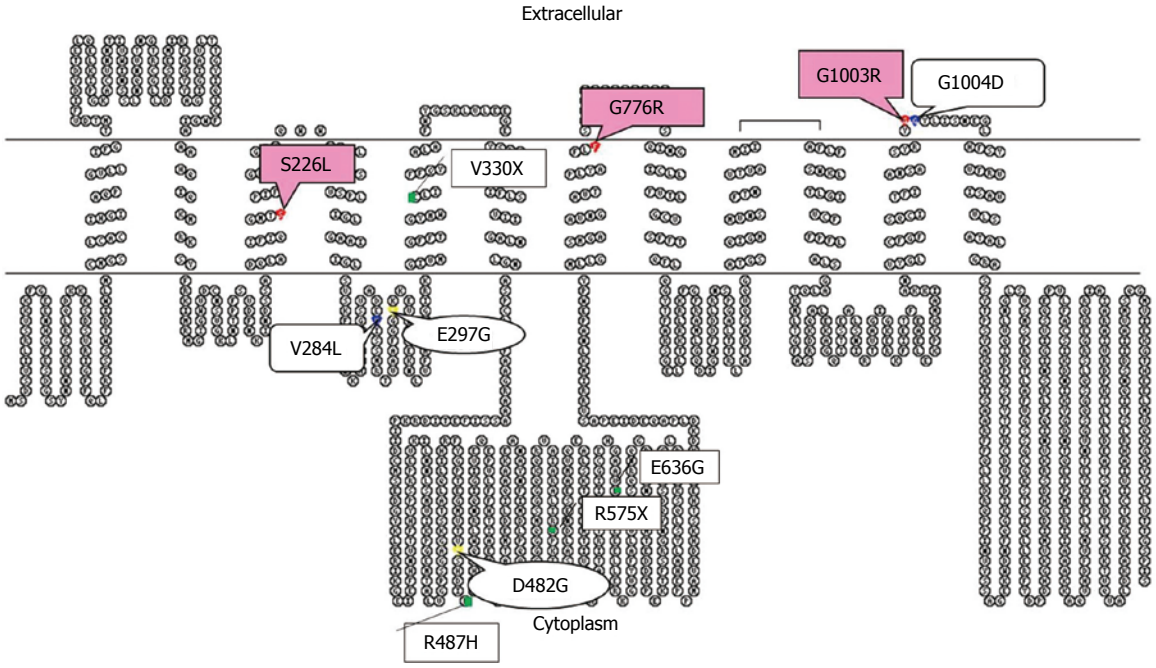

Figure 4 Putative secondary structure of bile salt export pump generated with the TOPO program (http://www.sacs.ucsf.edu/TOPO-run/wtopo.pl). Mutations are represented in red for mutations in patient 1 and 2, green for Japanese, blue for Taiwanese, and yellow for common European mutations. Bile salt export pump alterations in the present study were located at and near the transmembranous space, which was different from most of the mutations from Chinese, Japanese, Taiwanese, and European patients[2,5,7,12].

PFICs are developed by mutations of bile transporters, and the incidence of the mutation has not yet been established[9]. In addition, its incidence might be underestimated in Korea because diagnosis in a patient with suspected PFIC is often time consuming and expensive because of clinical methods such as clinical courses, laboratory findings, pathologic examination, and genetic analysis. Differentiation between PFIC1 and PFIC2 is difficult based on clinical manifestations and pathologic findings because of their marked clinical overlap[1]. PFIC1 patients usually present with more diverse extrahepatic symptoms, including diarrhea, than patients with PFIC2[10]. The patients did not show diarrhea or pruritus, but patient 1 did develop gallstones and HCC.

To the best our knowledge, Asian reports on PFIC2 are relatively lacking in comparison to Western reports[2,4-7]. However, reports on mutations of BSEP in Asian patients with PFIC2 are increasing by denaturing high performance liquid chromatography, high-resolution melting analysis, and direct sequencing. The reported Asian BSEP mutations were as follows: R575X, E636G, R487H, and V330X in Japanese patients; 1 bp deletion (position 1145), V284L, and G1004D in Taiwanese patients; and 20 mutations, including A167T, in Chinese patients[4,7,11,12]. Of the hundreds of BSEP mutations, E297G and D482G were the most common mutations in European patients[13]. Most of the previously reported mutations in Chinese and Europeans are located in the canalicular cytoplasm[2,4,12]. However, three different missense mutations (S226L, G1003R, and G775R) in the present study were located at and near the transmembranous (TM) part of BSEP (Figure 4). The TM alterations of BSEP in Korean infants might be due to ethnic differences. Further study, however, is needed because the number of mutations in the Korean patients with PFIC2 was low.

There was no mutation of *ABCB11* in patient 3, 4, and 5. TPJ2 mutations can also cause a PFIC2 like phenotype and further genetic analyses of TPJ2 are necessary[14]. Patient 5 showed elevated GGT levels and decreased serum total bilirubin in a relatively short clinical course. Therefore, the possibilities of PFIC1 and PFIC2 are less, but careful follow-ups are essential with the possibility of benign recurrent intrahepatic cholestasis.

Compound heterozygotes of 677C>T (S226L) and 3007G>A (G1003R) in patient 1, and one missense mutation of 2296G>A (G766R) in patient 2 were noted (Figure 3). The mutations were predicted to be damaging or deleterious to BSEP, based on the clinical course of the patients and the results of PolyPhen-2 and SIFT program. Unfortunately, the authors could not perform genetic analyses of *ABCB11* of the parents.

HCC developed in patient 1 (Figures 2 and 3A). Knisely *et al*[15] reported BSEP dysfunctions in 10 patients who presented with HCC under the age of 5 years. Chronic intrahepatic bile acids or suppressed DNA ligase by protein dysfunctions were suggested previously[16,17], but specific factors contributing to the development of HCC have not been evident. Cholangiocarcinoma and hepatoblastoma in patients with PFIC2 has also been reported[18,19]. The level of serum alpha fetoprotein (AFP) are high in > 60%

of patients with HCC and hepatoblastoma, and AFP increased markedly to 204000 ng/mL at 21 mo of age in patient 1. In patient 5, a significantly high level of AFP was noted on the first laboratory examination. There was no evidence of hepatic mass on liver ultrasonography (USG) and inborn errors of metabolism on laboratory examination. The level of AFP was decreased to 530000 ng/mL after 1 mo. The decline of AFP and no occurrence of hepatic mass on liver USG could rule out the development of hepatic tumor in patient 5. Therefore, the increment of serum AFP and hepatic imaging can be useful modalities for early detection of hepatic tumors in a patient with PFIC2.

Various degrees of periportal fibrosis, inflammatory cell infiltrates, intracytoplasmic and intracanalicular cholestasis, giant cell transformation, and bile ductular proliferation on the pathologic examinations were noted in the present study (Figure 2). Bile duct paucity or bile ductular proliferation was not a typical finding in patients with PFIC, but pathologic findings might depend on the clinical moment when the biopsy was performed. Pathologic examinations in the present study did not show typical findings for PFIC because our hepatic specimens were obtained at the time of liver transplantation, except in patient 5. Pathologic differentiation from PFIC1 to PFIC2 depends on the severity of the aforementioned findings and characters of canalicular bile salts. Coarse granular bile salts can suggest PFIC1, while amorphous and filiform bile salts under electron microscopic examination can suggest PFIC2[1]. However, pathologic differentiation seems to depend significantly on clinical moments for biopsy and experience or skill of the pathologist.

In conclusion, the present study is the first report on Korean infants with PFIC, including early onset HCC, living donor liver transplantations, and novel mutation and ethnic differences of *ABCB11*. We tentatively suggest the suspicion of PFIC1 or PFIC2 when children are suffering from chronic intrahepatic cholestasis with normal GGT and without other associated anomalies from their infantile periods, regardless of family history. Early genetic analysis for PFIC1 or PFIC2 might be helpful to diagnose, predict prognosis, and make an early treatment plan.

COMMENTS

Background

Because progressive familial intrahepatic cholestasis (PFIC)2 features rapid progression to liver cirrhosis and hepatic failure within the first decade, rapid diagnosis, management, and prediction of prognosis are important. The authors investigated the clinical profiles of Korean infants with PFIC, performed mutation analysis on the *ABCB11* gene, and reviewed the differences between Korean and other previous mutations.

Research frontiers

In the present study, a novel mutation of *ABCB11* in Korean infants with PFIC2 and the sites of amino acid alterations were different from those previously identified in Europeans.

Innovations and breakthroughs

The authors report a rare PFIC2 in Korean patients and its genetic novel mutations. These mutations affected amino acid substitutions of BSEP, and the sites were different from previous reports. The number of mutations, however, was low.

Applications

The described novel mutations of *ABCB11* in Korean infants with PFIC2 might be helpful to understand racial differences in the future. However, further study is warranted, including a larger single nucleotide polymorphism database of Koreans.

Peer-review

There were concerns about the number of mutations in Korean infants with PFIC2. However, the focus of the manuscript is potentially interesting, and the present report is significant because of the rarity in character of PFIC2, especially in Asian groups.

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ORIGINAL ARTICLE

Retrospective Study

Primary hepatic epithelioid angiomyolipoma: A malignant potential tumor which should be recognized

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Author contributions: Liu J and Zhang CW contributed equally to this work; Liu J collected and analyzed the data, and drafted the manuscript; Zhang CW designed and supervised the study; Tao R revised the manuscript; Chen Y was responsible for pathological analysis; Shang MJ and Zhang YH provided analytical oversight; and Hong DF provided academic support; all authors have read and approved the final version to be published.

Institutional review board statement: The study was reviewed and approved by the Zhejiang Provincial People's Hospital Institutional Review Board.

Informed consent statement: The study participant provided informed written consent for this study.

Conflict-of-interest statement: We declare that we have no financial or personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled "Primary hepatic epithelioid angiomyolipoma: A malignant potential tumor which should be recognized".

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at zcw1989@sina.com. Participants gave informed consent for data sharing.

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Abstract

AIM: To improve the clinical diagnosis and recognition of hepatic epithelioid angiomyolipoma (HEAML).

METHODS: Four cases of primary HEAML were confirmed based on the pathology archive system in our hospital from January 2009 to November 2015. The general state, clinical symptoms, imaging manifestations, histological results and immunohistochemistry of these patients were retrospectively reviewed and analyzed. Studies of HEAML published in the last 15 years were collected from PubMed and MEDLINE to summarize the clinical symptoms, imaging characteristics, pathological features and management of HEAML.

RESULTS: Four cases of primary HEAML were retrieved from our archives. These included three female patients and one male patient, with a mean age of 41.8 ± 11.5 years (ranging from 31 to 56 years). The mean

tumor size was 7.3 ± 5.5 cm (ranging from 3.0 to 15 cm). In the contrast-enhanced imaging, the tumor was obviously enhanced in the arterial phase, but enhanced continuously or exhibited a slow-density masse during the venous and delayed phases. Histologically, the tumors mainly consisted of epithelioid cells that comprised approximately 95% of the total neoplastic mass. Although no metastases occurred in our patients, pathological studies revealed necrosis, mitotic figures and liver invasion in two patients, which indicates aggressive behavior. Immunohistochemical staining revealed that human melanoma black 45 (HMB-45) and Melan-A were positive in 4 cases. We only identified 81 cases with primary HEAML, including our present patients, from 26 articles available from PubMed and MEDLINE. The majority of the papers were published as case reports. Only 5 (5/75, 6%) cases were associated with tuberous sclerosis complex (TSC). More than half (35/66) were discovered incidentally upon physical examination. Approximately 65% (22/34) of the patients were misdiagnosed with HCC or other tumors before surgery. Approximately 10% (8/81) of the patients with HEAML had recurrence or metastasis after surgery, which was a very high and alarming rate.

CONCLUSION: HEAML is a very rare primary hepatic tumor that is often misdiagnosed before surgery. Patients should be followed closely after surgery because of its malignant potential.

Key words: Epithelioid angiomyolipoma; Imaging; Liver; Immunohistochemical staining; Human melanoma black 45

Core tip: Hepatic epithelioid angiomyolipoma (HEAML) is very rare tumor that is often misdiagnosed because of atypical symptoms and imaging manifestation. The diagnosis can be made based upon characteristic pathological and immunohistochemical criteria. Traditionally, it is thought to be a benign tumor and is therefore largely ignored. Thus, it is important to improve the recognition of HEAML. This is the first article to analyze the clinicopathological data and imaging results of HEAML comprehensively by combining our four patients with other cases reported worldwide. In fact, HEAML has malignant potential and should be followed closely after surgery.

Liu J, Zhang CW, Hong DF, Tao R, Chen Y, Shang MJ, Zhang YH. Primary hepatic epithelioid angiomyolipoma: A malignant potential tumor which should be recognized. *World J Gastroenterol* 2016; 22(20): 4908-4917 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i20/4908.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i20.4908

INTRODUCTION

Angiomyolipoma (AML) is a kind of solid tumor that contains varying proportions of fat, smooth muscle cells, and blood vessels. Depending upon the dominant cell type, AML can be subcategorized into epithelioid, spindle, and intermediate forms. The former are perivascular epithelioid cells (PECs), including AML, lymphangiomyomatosis, and clear cell "sugar" tumor of the lungs[1]. Epithelioid angiomyolipoma (EAML) is a rare and special type of angiomyolipoma that most commonly occurs in the kidney, lung, heart, mediastinum, retroperitoneum, and vagina. Hepatic EAML (HEAML) was first reported by Yamasaki *et al*[2], and so far no more than 80 cases have been reported worldwide. HEAML, which was generally considered benign in the past, has malignant potential according to these reports[3]. There are no unique clinical symptoms of HEAML, which leads to confusion with other types of hepatic tumors easily[4]. Therefore, it has a very high rate of misdiagnosis. Here, we retrospectively reviewed the clinicopathological features of HEAML patients based on PubMed and MEDLINE data, including our four patients, who were finally diagnosed with HEAML through pathology and immunohistochemistry. The aim of our study was to improve the rate of accurate preoperative diagnosis and the degree of recognition of this rare tumor.

MATERIALS AND METHODS

Four cases of primary HEAML were confirmed based on the pathology archive system in our hospital from January 2009 to November 2015. The general state, clinical symptoms, imaging manifestations, histological results and immunohistochemistry of these patients were retrospectively reviewed. Important histological parameters including the diameter of the main tumor, the number of tumor nodules, cytological atypia, coagulative necrosis, mitotic count, liver invasion and vascular invasion were collected. Immunohistochemical staining for HMB-45, Melan-A, smooth muscle actin (SMA), HepPar-1, S-100, CK and fetoprotein (AFP) was repeated to verify the diagnosis. Follow-up data were obtained from the clinical records.

Articles about primary HEAML (excluding PEComa) were collected from January 2000 to November 2015 from PubMed and MEDLINE, and non-English publications were included. Clinical data were retrieved from the articles including sex, tumor size, case number, location of tumor, clinical presentation, preoperative diagnosis, association with TSC, treatment and prognosis.

SPSS (version 15.0 for Windows) software was used for statistical analyses. The results are presented as the mean ± SD or median.

Table 1 Clinicopathologic data of 4 cases of hepatic epithelioid angiomyolipoma

Case	Sex/age	Size (cm)	Location of tumor in the liver	Clinical presentation	Fat	Details of imaging findings	Preoperative diagnosis	Pathologic features					
								Satellite tumor	Cytologic atypia	Mitotic count	Coagulative necrosis	LI	VI
1	M/34	15.0	L	Abdominal pain and fever	No	Arterial phase enhancement and washout at portovenous phase (CT)	HCC	+	+	+	+	+	-
2	F/46	3.5	L	None	No	Arterial phase enhancement and washout at portovenous phase (CT)	HCC	-	-	-	-	-	
3	F/31	3.0	R	None	No	Arterial phase enhancement and washout at portovenous phase (CT)	HCC	-	+	+	+	+	-
4	F/56	7.5	L	None	No	Arterial phase enhancement and no delayed washout at portovenous phase (MRI)	HCA	-	-	-	-	-	-

LI: Liver invasion; VI: Vascular invasion; HCC: Hepatocellular cancer; HCA: Hepatocellular adenoma. Size of the main tumor nodule.

RESULTS

The clinicopathological features of the 4 cases are summarized in Table 1. All 4 cases received surgical resection. The pathological and histological diagnosis of HEAML was verified in all 4 cases. Three patients were female and one patient was male. The mean age was 41.8 ± 11.5 years (ranging from 31 to 56 years). The male patient was rushed to the emergency room because of upper abdominal pain and fever, whereas the other 3 patients presented with liver masses on ultrasound but without symptoms or signs upon physical examination. The medical histories of all four patients were normal. Tests for the hepatitis C virus antibody and hepatitis B virus surface antigen were all negative, and there was no evidence of tuberous sclerosis. The results of the liver function test, routine blood test, serum AFP and carbohydrate antigen199 (CA199) were normal for our 4 patients with the exception of the male patient, who had elevated alkaline phosphatase; however, this patient tested negative for bacterial infection, and a fever caused by the tumor was considered first. All of the cases presented with intraparenchymal tumors, and the mean tumor diameter was 7.3 ± 5.5 cm (ranging from 3.0 to 15 cm). In the three female patients, the tumor presented as a single lesion. One tumor was located in the right lobe, while the other two tumors were located in the left lobe. In contrast, the male patient presented with three tumor lesions in bilateral liver lobes. Detailed imaging results were available for the four patients. Dynamic contrast-enhanced imaging showed two different presentations. The computed tomography scan of the male patient showed multiple low-density masses in the plain phase (Figure 1A), with the biggest lesion, measuring 15.0 cm × 12.0 cm × 10.0 cm in size, in the left hepatic lobe. In the arterial phase, strong contrast enhancement with low density in the center was observed (Figure 1B). The tumors had almost washed out the contrast agent except for a weak contrast-enhanced effect in the center that was perhaps induced by arterioportal venous shunting in the portal phase (Figure 1C). In the delayed phase, the tumors exhibited low density in comparison with the liver tissue (Figure 1D). The imaging of patients 2 and 3 showed similar manifestations. However, for patient 4, the foci had showed boundaries and high signal in T2WI (Figure 2A) but low signal in the plain phase (Figure 2B). A significantly and uniformly enhanced mass during the arterial phase was observed (Figure 2C). However, different from other cases, this mass was enhanced continuously during the venous and delayed phases (Figure 2D). Based on the available clinical findings, a preoperative diagnosis of hepatocellular adenoma (HCA) or HCC was made. None of the cases presented with extrahepatic metastasis, and they were diagnosed as primary liver tumors with no evidence of other organ involvement. Extended left lobectomy and laparoscopic hepatectomy were performed, respectively, because of the uncertain nature.

With respect to the pathological findings, gross examination revealed that the external surface of the mass was smooth and brownish in color. Sections revealed well-circumscribed, non-encapsulated tumors. The largest tumor was multiloculated with amorphous

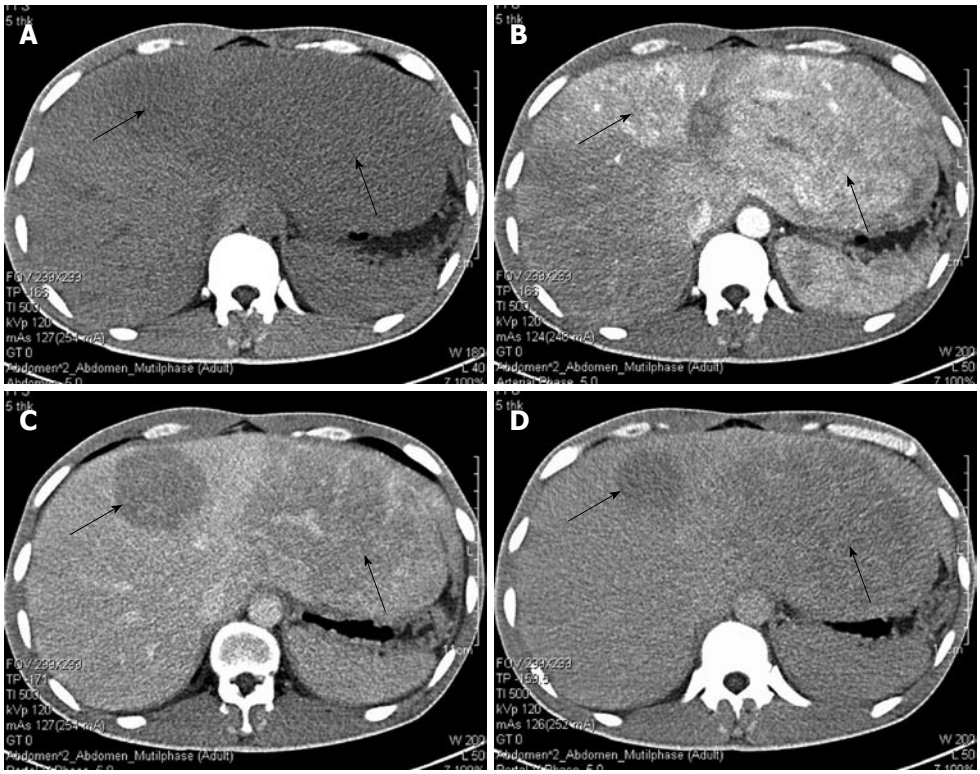

Figure 1 Computed tomography manifestation of the male patient. A: Low-density masses in the plain phase (black arrows); B: In the arterial phase, a strong contrast-enhancing effect with low-density in center was observed; C: In the portal phase, tumors had almost washed out the contrast agent, but a weak contrast-enhancing effect was sustained; D: Low-density masses in the delayed phase.

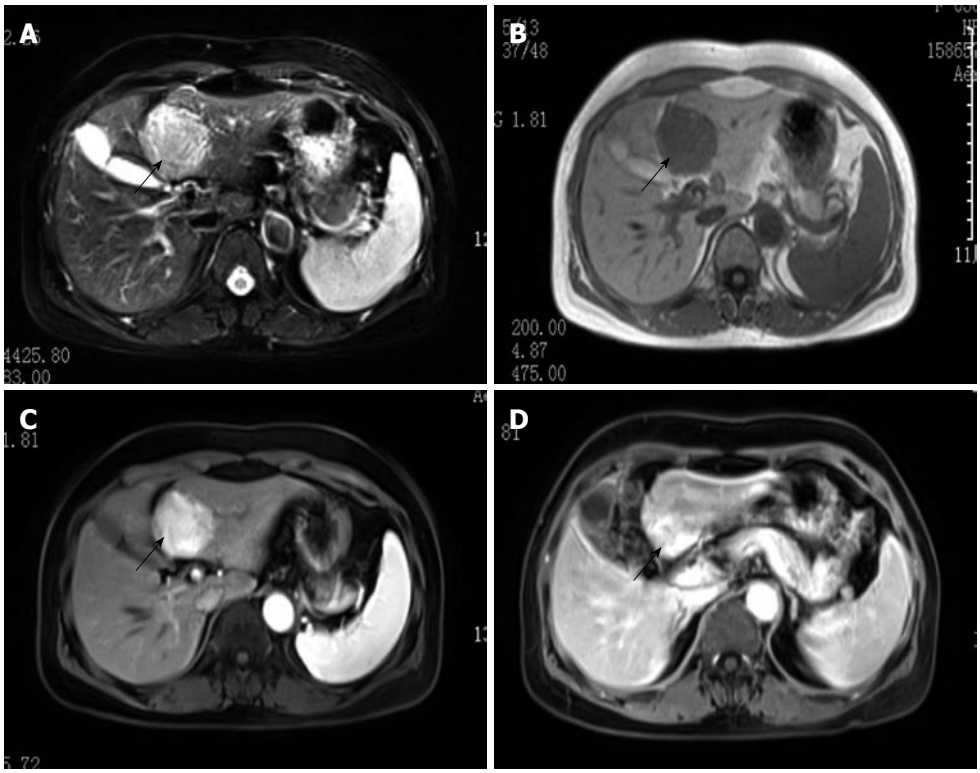

Figure 2 Magnetic resonance imaging manifestation of the female patient. The tumor had clear boundaries and showed high signal on T2WI (A, black arrow) and low signal in the plain phase (B); Hyperenhancement in the arterial phase (C) and delayed phase (D).

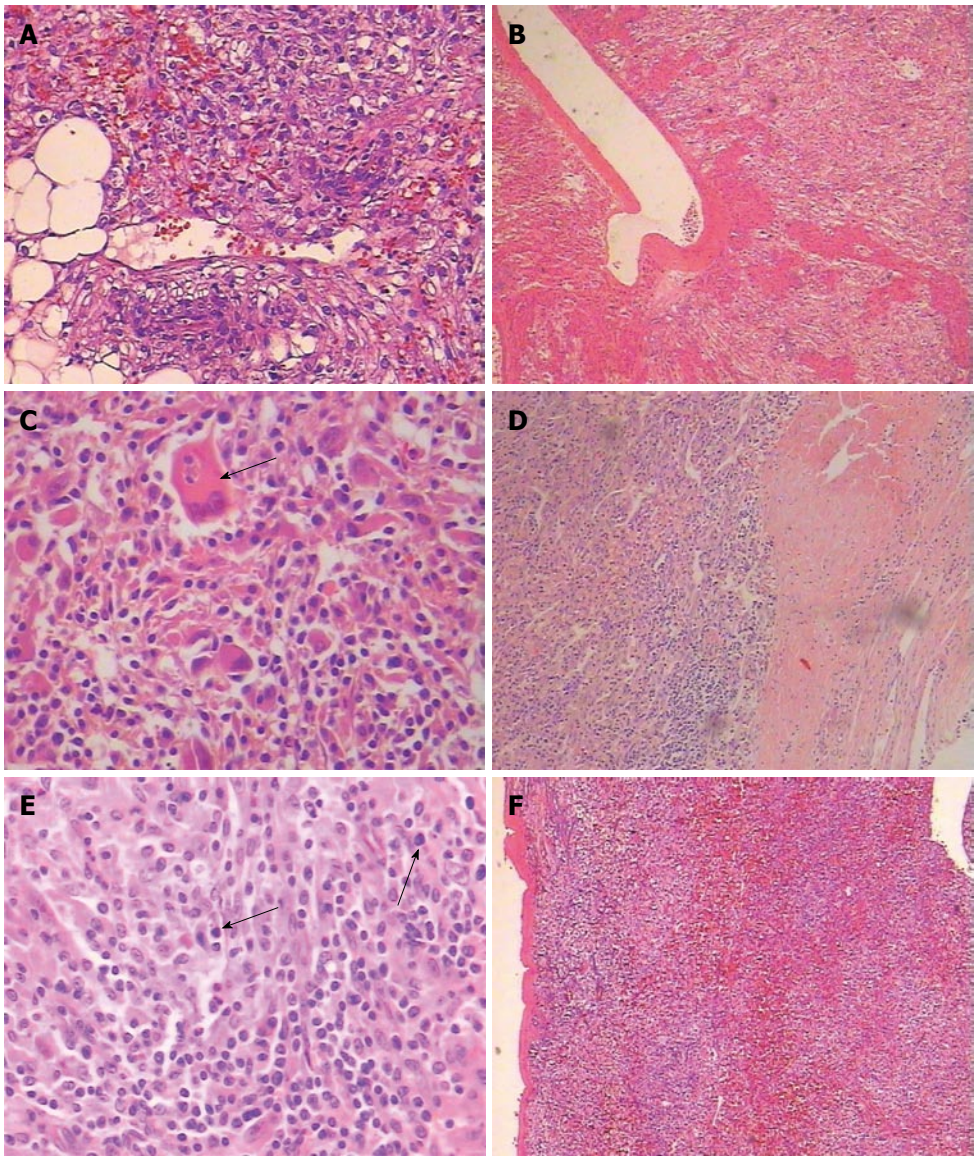

Figure 3 Histology of hepatic epithelioid angiomyolipoma. A: The tumor comprised of sheets of large polygonal cells with abundant granular eosinophilic cytoplasm (HE, × 200); B: The tumor cells were arranged radial around blood vessels (HE, × 40); Microscopic features signifying aggressive behavior were observed: C: Cytologic atypia (black arrow) (HE, × 200); D: Coagulative necrosis (HE, × 40); E: Increased mitotic count (black arrow) (HE, × 200); F: Tumors mainly consisted of epitheli¬oid cells that comprised approximately 95% of the total neoplastic mass (HE, × 40).

necrotic tissue and hemorrhagic fluid. The male patient had the other two satellite tumor nodules whose size was 1 cm and 2 cm, respectively. Microscopically, the tumor was comprised of sheets of large polygonal cells with abundant granular eosinophilic cytoplasm in areas with typical features of HEAML (Figure 3A). A radial arrangement around blood vessels was observed (Figure 3B). Microscopic features including cytologic atypia (Figure 3C), coagulative necrosis (Figure 3D) and increased mitotic count (Figure 3E) were observed in two cases, which indicates aggressive behavior. All tumors mainly consisted of epithelioid cells that comprised approximately 95% of the total neoplastic mass (Figure 3F). Tumors also contained a few spindle myoid cells, mature fat, and thick-walled vasculature. Immunohistochemical analysis revealed that 4 patients were positive for the melanocytic markers HMB-45 (Figure 4A), Melan-A (Figure 4B), SMA (Figure 4C) and VIM (Figure 4D), but negative for S-100 (Figure 4E), CK (Figure 4F), AFP (Figure 4G) and HepPar-1 (Figure 4H). The diagnosis was corrected to HEAML based on the above evidence. The median follow-up period was 30 months (ranging from 2 to 72 mo). All patients were alive with no evidence of recurrence at the time of review.

Our search of PubMed and MEDLINE confirmed a total of 81 cases (including our four cases) with primary HEAML from 26 articles[2,3,5-27] (Table 2). The majority of the cases were single case reports with the exception of 6 publications that reported more than 5 cases per article. Even so, those articles only focused on the pathological or imaging presentations. These limited results reflect the overall poor recognition of HEAML. Summarizing these available reports is

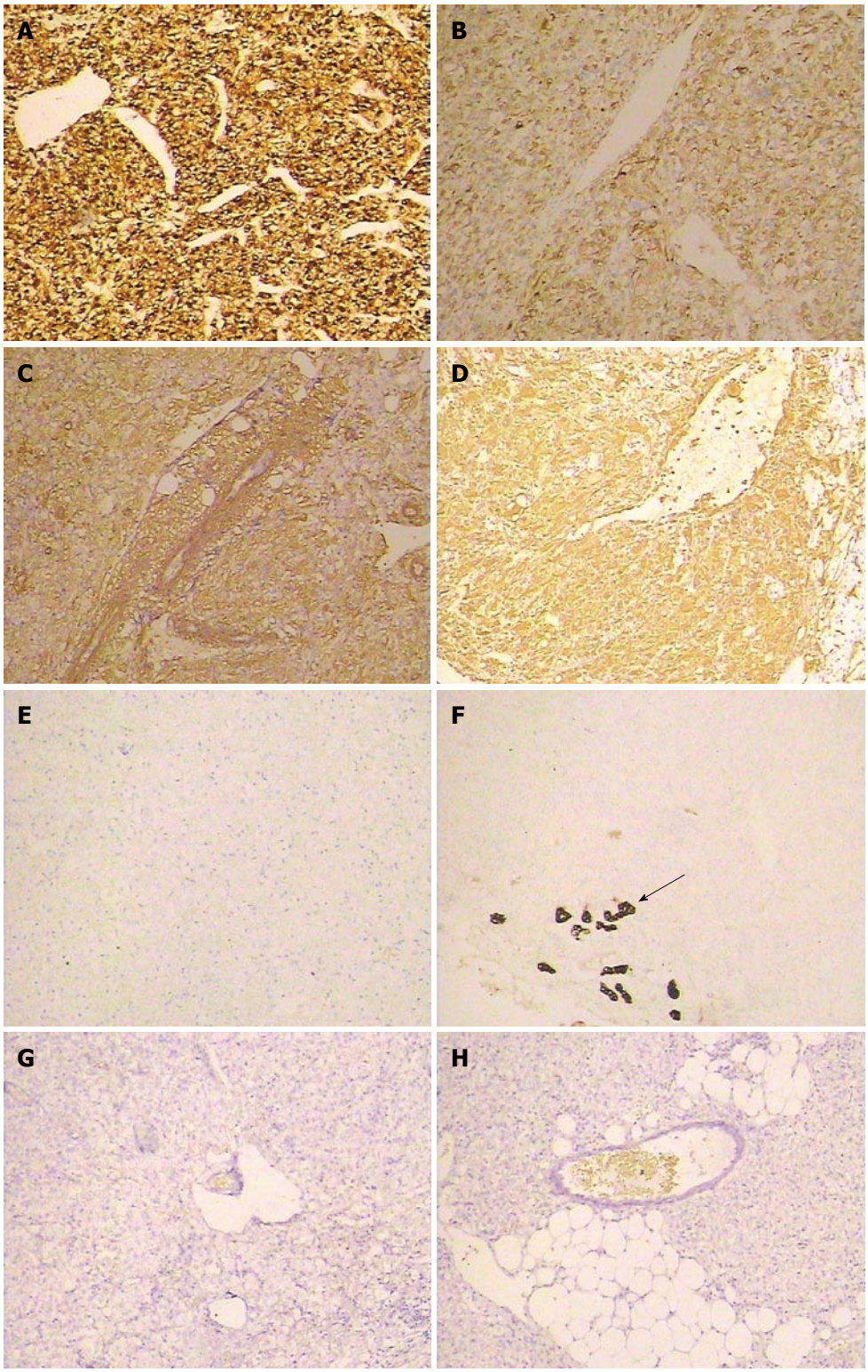

Figure 4 Immunohistochemical staining of hepatic epithelioid angiomyolipoma. The tumor cells were positive for melanocytic markers HMB-45 (A, × 40), Melan-A (B, × 40), SMA (C, × 40), and VIM (D, × 40), but negative for S-100 (E, × 40), CK (the arrow indicates the bile duct epithelium) (F, × 40), AFP (G, × 40), and Herpar-1 (H, × 40).

necessary to further promote the diagnosis of HEAML.

DISCUSSION

In all series of HEAML, more women suffered from HEAML than men, at a ratio of 5:1. Although some patients with HEAML had atypical gastrointestinal symptoms, including abdominal pain or distension, discomfort and vomiting, more than half (35/66, 53%) of the cases were discovered incidentally upon

Table 2 Summary of available reports of hepatic epithelioid angiomyolipoma from PubMed and MEDLINE

Year	Authors	Number (F/M)	Size (cm)	Location (L/R)	Clinical symptoms (*n*)	Diagnosis before surgery (*n*)	TSC (*n*)	Treatment	Recurrence/ metastasis (*n*)
2015	Our study	3/1	3.0-15.0	3/1	Abdominal pain and fever (1) No symptoms (3)	HCC (3) HCA (1)	None	Surgery	None
2014	Dai *et al*[24]	3/2	2.5-7.0	2/3	Abdominal pain (2) None (3)	HCC (4) FNH (1)	None	Surgery	None
2014	Tajima *et al*[25]	0/1	10.5	0/1	Abdominal pain	Hepatic AML	None	Surgery	None
2014	Zhou *et al*[27]	1/0	30.0	1/0	Abdominal discomfort	NA	None	Surgery	None
2014	Xu *et al*[26]	22/3	3.0-20.0	12/13	Abdominal pain (10) Abdominal distention (3) No symptoms (15)	HCC or other tumor	1	Surgery	2
2013	Occhionorelli *et al*[22]	1/0	8.0	1/0	Abdominal pain	NA	NA	Surgery	None
2013	Zhao *et al*[23]	3/2	0.6-9.7	2/3	No symptoms	NA	None	Surgery	NA
2013	Saito *et al*[21]	0/1	1.2	1/0	No symptoms	HCC	None	Surgery	None
2013	Lo *et al*[20]	5/0	1.2-25.0	1/4	Abdominal distention (1) Abdominal discomfort (1) Epigastralgia (1) None (2)	HCC (1) HCA (2) AML Liver tumor with uncertain nature	None	Surgery	None
2013	Ji *et al*[19]	6/0	5.0-9.5	5/1	None (5) Abdominal pain (1)	AML	None	Surgery and biopsy	NA
2012	Limaiem *et al*[18]	0/1	8.2	1/0	Abdominal pain	HCC	None	Surgery	None
2012	Agaimy *et al*[16]	1/0	2.0	1/0	Nausea	Metastatic adenocarcinoma or carcinoid	None	Surgery	None
2012	Xie *et al*[17]	1/0	3.4	0/1	Dyspnea	HCC	1	Biopsy	None
2010	Wen *et al*[15]	0/1	4.1	1/0	None	HCC	NA	Surgery	NA
2009	Leenman *et al*[13]	1/0	6.0	1/0	Abdominal pain	NA	NA	Surgery	NA
2009	Xu *et al*[14]	10/0	1.5-10.0	6/6	NA	NA	1	Surgery	2
2009	Alatasis *et al*[12]	1/0	11.0	Multiple	None	HCC	1	Biopsy	NA
2008	Deng *et al*[11]	0/1	18.0	0/1	Abdominal pain	AML	None	Surgery	1
2007	Khalbuss *et al*[10]	1/0	12.0	1/0	Abdominal pain	Adenoma or hamartoma	1	Surgery	None
2006	Rouquie *et al*[9]	1/0	7.0	1/0	None	NA	None	Surgery	None
2004	Tryggvason *et al*[8]	1/0	6.0	1/0	Abdominal pain	NA	None	Surgery	None
2004	Mizuguchi *et al*[7]	1/0	NA	0/1	None	AML	NA	Surgery	1
2000	Savastano *et al*[6]	1/0	1.2	1/0	NA	NA	NA	Surgery	None
2000	Flemming *et al*[5]	3/0	1.0-20.0	2/2	NA	HCC	None	Surgery (2) Biopsy (1)	1
2000	Yamasaki *et al*[2]	1/0	2.0	0/1	None	NA	None	Surgery	None
2000	Dalle *et al*[3]	1/0	15.0	0/1	Nausea and loss of appetite	HCC	NA	Biopsy	1

F/M: Female/male; L/R: Left/right; TSC: Tuberous sclerosis complex; HCC: Hepatocellular cancer; HCA: Hepatocellular adenoma; FNH: Focal nodular hyperplasia; AML: Angiomyolipoma; NA: Not available.

physical examination based on the available data and were similar to three female patients in our research. One of our patients had a fever due to the central necrosis in the large size of the tumor. Rupture and hemorrhage were reported as the first symptoms in a few cases[22,25]. Abnormal liver function was frequently observed in patients with larger tumor size. Tumor size varied considerably, with lesions ranging from a few millimeters to as large as 30 cm having been reported. In general, bigger tumors have greater malignant potential. There was no difference regarding the location of the tumor in either the left or right lobe (44:39). Some reports showed that 26%-32% of AML patients had associated TSC[28,29]; however, interestingly, this ratio was less than 5% in China[26]. Among 75 patients who had valid data, only 5 (5/75, 6%) were associated with TSC, which was similar to the domestic study. All four cases in our report were solitary tumors without TSC. It was confirmed that approximately 50% of TSC patients had AML, but approximately 80% of the patients with AML were sporadic cases, which were not related to TSC.

HEAML always presents with less typical imaging manifestations, especially when smooth muscle and vascular components dominated in tumor as seen in the less fat types, which leads to confusion with other hepatic tumors easily and making a correct diagnosis very difficult before surgery. In dynamic enhanced CT or magnetic resonance imaging, multiple manifestations of HEAML were observed[21,23,24]. Most were obviously enhanced in the early arterial phase but showed low density in the portal venous phase and delayed phase, which has been confirmed by our research and by previous reports in the literature. Similar imaging signs could be observed in other hypervascular hepatic lesions, just like HCC. It is very

difficult to discriminate between HEAML and HCC; 60% of patients with HEAML were misdiagnosed with HCC before surgery in 81 cases. According to the pathology, the so-called "false capsule" of HEAML, different from the real capsule of HCC, was just formed by the compression of the surrounding liver tissues, and had no histological structure in fact. Although the excretion of the contrast medium was relatively slow on the imaging in HEAML, this difference was not enough to distinguish the HCC or HEAML, especially in large tumors with central necrosis or hemorrhage. Focal nodular hyperplasia (FNH) always showed a central scar, which is a characteristic sign and could be an important basis in the differential diagnosis. In addition, FNH presented delayed enhancement on enhancement scans, which was also different from most HEAML. One patient in our study was diagnosed with hepatic adenoma during hospitalization because of enhancement in all phases on enhancement scans. Other fat rich tumors such as lipoma show almost no enhancement on imaging scans because of poor blood supply.

Pathology is the only definite diagnostic criteria. The gross observation of HEAML is not characteristic; most are solitary but multifocal tumors, such as cystic degeneration, have been reported in several case reports. Its morphological features under the microscope were revealed by Xu *et al*[26] by analyzing 25 cases of HEAML. It was characterized by marked cytological atypia; relatively rare mitotic figures; radial distribution of tumor cells around the thin-walled blood vessels or muscular vessels; and the presence of common multinucleated giant cells and large ganglion-like tumor cells. Although the presence of epithelioid cells is important for the diagnosis of EAML, the ratio is still under debate. Aydin *et al*[29] thought that it could be defined as an EAML if epithelioid cell components were greater than 10%, but most scholars believed that this standard was too low and should be as high as 50%, or even more than 90%. In our patients, the epithelial cells of EAML reached more than 95%. Apparently, more cases and follow-up results are needed to reach a unanimous conclusion.

Renal EAML has malignant potential and may metastasize to the lymph node, liver, lung, or bone in approximately one-third of cases. Poor outcome is considered when necrosis, mitotic figures, or a plastic nucleus are observed in pathological studies. Nese *et al*[30] showed that a carcinoma-like growth pattern and extrarenal extension and/or renal vein involvement were significant independent prognostic factors in a multivariate analysis. Brimo *et al*[31] summarized the pathological characteristics of renal EAML progression: (1) $\geqslant$ 2 mitotic figures per 10 high-power field; (2) atypical mitotic figures; (3) $\geqslant$ 70% of atypical epithelioid cells; and (4) necrosis. The presence of 3 or more features was highly predictive of malignant behavior. Faraji *et al*[32] also showed that marked cytological atypia and extensive tumor necrosis were related to the progression of EAML. Although HEAML is considered to be a benign tumor in several series of case reports, 8 cases of malignant HEAML have been reported[3,5,7,11,14,26], and there is a lack of evidence to determine whether the same prognostic parameters of renal EAML are applicable to HEAML.

As a member of the PEComa family, the immunological phenotype of HEAML has the characteristics of bidirectional differentiation of melanoma cells and smooth muscle cells. The tumor cell is positive for the expression of cell markers including MART-1, HMB45, Melan A and SMA, but negative for all epithelial markers including EMA and S-100. This is the most important criterion for the differential diagnosis of HEAML. Those tumor cells are also negative for typical markers of HCC, including AEP, HepPar 1 and canalicular polyclonal CEA. HEAML is sometimes misdiagnosed as malignant melanoma because of the differentiation of melanocytes; however, primary hepatic melanoma is very rare, and the tumor cells are positive for S-100 but negative for smooth muscle cell markers based on immunohistochemical analysis.

Based on previous reports, surgery is the only effective way to cure HEAML; however, biopsies were also used in a few cases with the risk of tumor growth and metastasis. In one case, the tumor volume increased from 760.8 cm^3 to 1967.8 cm^3, a 2.6-fold increase during the 102 d after HEAML was diagnosed by biopsy[7]. A metastatic mass in the right lower quadrant and portal vein thrombosis were suspected in another biopsy case. Approximately 10% (8/81) of patients with HEAML had recurrence or metastasis after surgery, which was a very high and alarming rate. Although no recurrence or metastasis occurred in our study, pathological studies still show necrosis, mitotic figures and liver invasion in two patients, which indicates aggressive behavior. To be vigilant, although the majority of AMLs always are considered as benign tumors for their biological behavior, the potential risk of malignant changes of HEAML needs to be noticed and should be followed rigorously after surgery.

COMMENTS

Background

Hepatic angiomyolipoma (HAML) is a rare benign tumor that belongs to a family of tumors that have collectively been called "PEComa". As a specific form, the hepatic epithelioid angiomyolipoma (HEAML) has malignant potential and is often misdiagnosed because of atypical symptoms and imaging manifestations. Characteristic pathological and immunohistochemical features are the diagnostic criteria. Therefore, it is important to improve the recognition of HEAML.

Research frontiers

In the past15 years, there have been scattered reports of HEAML, and the majority of those were case reports. In addition, those articles only focused on the pathology or imaging aspects, respectively. Therefore, more cases need to be collected and summarized, and more attention should be paid to this disease.

Innovations and breakthroughs

This is the first study to summary the clinical symptoms, imaging manifestations and pathological features of HEAML by retrospectively analyzing 81 cases from 26 articles, including our four patients, to improve the recognition of HEAML and reduce the misdiagnosis of this disease.

Applications

By understanding the characteristics of the clinical symptoms, imaging manifestations and pathological features of HEAML, this study could help us to improve confidence in the diagnosis of HEAML, especially in the differential diagnosis with other solid tumors in the liver, such as hepatic cellular cancer and adenoma.

Peer-review

In this manuscript, the authors analyze and summarize the clinical symptoms, imaging manifestations and pathological features of patients with HEAML by collecting all 81 cases to improve the rate of accurate diagnosis. No similar report has been published before. It will be helpful for scholars to obtain knowledge of this disease.

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ORIGINAL ARTICLE

Observational Study

Efficacy of peroral endoscopic myotomy *vs* other achalasia treatments in improving esophageal function

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Abstract

AIM: To assess and compare the esophageal function after peroral endoscopic myotomy (POEM) *vs* other conventional treatments in achalasia.

METHODS: Chart review of all achalasia patients who underwent POEM, laparoscopic Heller myotomy (LHM) or pneumatic dilation (PD) at our institution between January 2012 and March 2015 was performed. Patient demographics, type of achalasia, prior treatments, pre- and post-treatment timed barium swallow (TBE) and high-resolution esophageal manometry (HREM) findings were compared between the three treatment groups. Patients who had both pre- and 2 mo post-treatment TBE or HREM were included in the final analysis. TBE parameters compared were barium column height, width and volume of barium remaining at 1 and 5 min. HREM parameters compared were basal lower esophageal sphincter (LES) pressures and LES-integrated relaxation pressures (IRP). Data are presented as mean ± SD, median [25th, 75th percentiles] or frequency (percent). Analysis of variance, Kruskal-Wallis test, Pearsons χ^2 test and Fishers Exact tests were used for analysis.

RESULTS: A total of 200 achalasia patients were included of which 36 underwent POEM, 22 underwent PD and 142 underwent LHM. POEM patients were older (55.4 ± 16.8 years *vs* 46.5 ± 15.7 years, $P = 0.013$) and had higher BMI than LHM (29.1 ± 5.9 kg/m^2 *vs* 26 ± 5.1 kg/m^2, $P = 0.012$). More number of patients in POEM and PD groups had undergone prior treatments compared to LHM group (72.2% *vs* 68.2% *vs* 44.3% respectively, $P = 0.003$). At 2 mo post-treatment, all TBE parameters including barium column height, width and volume remaining at 1 and 5 min improved significantly in all three treatment groups ($P = 0.01$ to $P < 0.001$) except the column height at 1 min in PD group ($P = 0.11$) . At 2 mo post-treatment, there was significant improvement in basal LES pressure and LES-IRP in both LHM (40.5 mmHg *vs* 14.5 mmHg and 24 mmHg *vs* 7.1 mmHg respectively, $P < 0.001$) and POEM groups (38.7 mmHg *vs* 11.4 mmHg and 23.6 mmHg *vs* 6.6 mmHg respectively, $P < 0.001$). However, when the efficacy of three treatments were compared to each other in terms of improvement in TBE or HREM parameters at 2 mo, there was no significant difference ($P > 0.05$).

CONCLUSION: POEM, PD and LHM were all effective in improving esophageal function in achalasia at short-term. There was no difference in efficacy between the three treatments.

Key words: Achalasia; Dysphagia; Heller myotomy; Peroral endoscopic myotomy; Manometry; Pneumatic dilation

Core tip: This study evaluated and compared the efficacy of peroral endoscopic myotomy with laparoscopic Heller myotomy and pneumatic dilation in improving esophageal function in achalasia. Esophageal function was objectively assessed by timed barium esophagram and high resolution manometry at 2 mo follow-up. The results demonstrate that all three treatment modalities are effective in improving esophageal function at short term follow-up and there was no difference in efficacy between the three treatment modalities.

Sanaka MR, Hayat U, Thota PN, Jegadeesan R, Ray M, Gabbard SL, Wadhwa N, Lopez R, Baker ME, Murthy S, Raja S. Efficacy of peroral endoscopic myotomy *vs* other achalasia treatments in improving esophageal function. *World J Gastroenterol* 2016; 22(20): 4918-4925 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i20/4918.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i20.4918

INTRODUCTION

Achalasia is a rare primary esophageal motility disorder, with an incidence of about 1 per 100000 per year[1]. The disease is characterized by aperistalsis of the esophageal body and impaired relaxation of the lower esophageal sphincter (LES), caused by progressive destruction and degeneration of neurons in the myenteric plexus. Typical symptoms of achalasia are dysphagia, regurgitation of undigested food, retrosternal pain, and weight loss. The disease is irreversible and all the current treatments of achalasia are aimed at palliation of symptoms[2]. Established treatment options include disruption of the LES by endoscopic pneumatic dilation (PD) and laparoscopic Heller myotomy (LHM). Both treatments are considered the "standard of care" and have similar excellent short-term results, as demonstrated in a large, randomized, controlled trial[3]. Because of submucosal fibrosis after treatment and the natural course of the disease, symptoms can recur, leading to a need for retreatment in some patients. LHM has been shown to provide more durable long-term symptom relief than PD and is considered the preferred treatment[4]. Recently, peroral endoscopic myotomy (POEM) is emerging as an alternative to LHM. POEM has the advantages of minimal invasiveness of an endoscopic procedure and the precision of a surgical myotomy[5].

Both PD and LHM improve parameters of objective esophageal function, such as LES pressures on high resolution esophageal manometry (HREM), esophageal emptying on timed barium esophagram (TBE) and esophagogastric junction (EGJ) distensibility[6]. Objective improvement in these parameters regardless of symptoms is predictive of long-term favorable response. For example, patients with LES-Integrated relaxation pressures (IRP) of > 10 mmHg after treatment were shown to have a significantly higher risk for retreatment during follow-up[7-9]. Vaezi *et al*[10] have shown that patients with incomplete esophageal emptying after PD on TBE had a 90% risk for treatment failure within 1 year, whereas the treatment success rate remained about 90% in patients with complete emptying. Therefore, these parameters are useful not only to objectively determine esophageal function post-treatment, but also for predicting the need for retreatments.

Since POEM is relatively new, only short- and intermediate-term treatment success rates are available. There were several studies that showed objective improvement in esophageal function assessed by HREM and TBE findings after POEM[11-15]. Bhayani *et al*[16], reported that improvement in HREM parameters after POEM was comparable to LHM. To date, there are several studies comparing the improvement in esophageal function between either PD and LHM or POEM and LHM. However, there are no studies comparing the outcomes between all three treatment modalities. Hence, the aim of this study was to compare objective improvement in esophageal function among achalasia patients who underwent POEM, LHM and PD at our institution.

MATERIALS AND METHODS

This study was approved by the Institutional Review Board at the Cleveland Clinic. We reviewed medical records of all adult achalasia patients who underwent one of the three treatment modalities at our institution between January 2012 and March 2015. A written informed consent was obtained from all patients prior to the treatments. Patient demographics, type of achalasia, prior treatments, pre- and 2-mo post-treatment TBE and HREM parameters were compared between the three treatment groups. All patients undergoing either POEM or LHM had TBE and HREM performed before and at two months post-treatment as part of our standard clinical practice. Most of the patients who underwent PD had TBE and HREM performed before and TBE alone performed at two months post-treatment.

LHM procedure

In our patients, LHM was performed with anterior approach and thoracic esophagus was mobilized and full-thickness myotomy was performed along distal 4-6 cm of esophagus and was extended 2-3 cm on to the gastric wall. Subsequently a partial anterior fundoplication (Dor fundoplicaiton) was performed. Patients underwent barium swallow study next day to exclude perforation and liquid diet was initiated and gradually advanced over the next few days.

PD procedure

A standard upper endoscopy was performed under sedation by monitored anesthesia care and esophagus was cleared of any residual food debris. A guidewire was placed into the antrum and under fluoroscopic guidance, and a Rigiflex balloon (Boston Scientific, MA, United States) of either 30 mm or 35 mm diameter was passed and positioned across the gastroesophageal junction and inflated for few seconds until the "waist" was obliterated. A 30 mm balloon was used when patients underwent PD for the first time, and a 35 mm balloon was used for patients undergoing subsequent PD. All patients underwent a barium swallow post-procedure to exclude a perforation and were discharged home on clear liquid diet with gradual advancement of diet.

POEM procedure

All POEM procedures were performed under general anesthesia in an operating room using standard steps as described by Inoue *et al*[5]. The steps were (1) creation of a submucosal tunnel starting approximately 12 cm proximal to the LES and extending distally to about 2-4 cm into the stomach side. The submucosal tunnel was usually created on anterior esophageal wall except in post-Heller patients in whom it was created on the posterior esophageal wall; (2) Myotomy of the circular muscle fibers starting 3-4 cm distally from the first incision and 2-4 cm into the stomach wall; and (3) Closure of the entry site of the submucosal tunnel by using endoscopic clips. Next day, patients underwent a soluble contrast swallow radiograph to exclude transmural perforations. If swallow study is unremarkable, patients were started on clear liquid diet, discharged home and were advised to advance diet gradually over the next 1-2 wk.

HREM procedure

HREM was performed by using the following protocol: a 36-channel, solid-state catheter system with high-fidelity circumferential sensors at 1-cm intervals was advanced through the nasal canal (Sierra Scientific Instruments Inc., Los Angeles, CA, United States). Pressure data of ten, 5 mL swallows of water were recorded and analyzed by using a dedicated computerized analysis system. All relevant parameters were analyzed according to the Chicago classification. Diagnostic criteria for achalasia were incomplete relaxation of LES (IRP > 15 mmHg) and aperistalsis of the esophageal body. Achalasia was classified into type I, if there was 100% peristasis without esophageal pressurization, type II if there was pan-esophageal pressurization > 30 mmHg in $\geqslant$ 20% of swallows and as type III when there were premature contractions in $\geqslant$ 20% of swallows.

TBE procedure

Patients were instructed to drink the maximum volume of dilute barium sulfate contrast (45% weight in volume) that they could tolerate without regurgitation or aspiration (mostly between 100 and 250 mL) over a period of 30 to 45 s. With the patient in upright position, radiographs of the esophagus were taken at 1 and 5 min after the last swallow. Height and width of the barium column were measured using a calibrated ruler. Estimated esophageal barium volume was calculated as a simple cylinder (πr^2 × height of barium column, r = barium width divided by 2).

Statistical analysis

Data are presented as mean ± SD, median (25^{th}, 75^{th} percentiles) or frequency (percent). A univariable analysis was performed to assess differences between treatment groups. Analysis of variance (ANOVA) or the non-parametric Kruskal-Wallis tests were used for continuous or ordinal variables and Pearson's chi-square tests were used for categorical factors. When the overall test suggested a difference between at least 2 of the groups, post-hoc comparisons were done at a significance level of 0.017 (0.05/3 tests) to adjust for multiple comparisons. In addition, analysis of covariance was performed to assess the association between treatment and outcomes while adjusting for possible confounders. For each outcome, a logarithm transformation ln[(y-1) + min(y)] was modeled as the dependent variable with age at time of treatment, body mass index (BMI) and having had previous treatments

Table 1 Patient characteristics *n* (%)

Factor	PD (*n* = 22)		LHM (*n* = 142)		POEM (*n* = 36)		*P* value
	n	Summary	*n*	Summary	*n*	Summary	
Age at diagnosis (yr)	22	47.5 ± 17.0	142	45.8 ± 15.6	36	52.6 ± 17.2	0.078[a]
Age at current treatment (yr)	22	50.3 ± 17.9	142	46.5 ± 15.7[3]	36	55.4 ± 16.8[2]	0.013[a]
Gender	22		142		36		0.19[c]
Female		11 (50.0)		71 (50.0)		12 (33.3)	
Male		11 (50.0)		71 (50.0)		24 (66.7)	
Ethnicity	22		141		36		0.85[d]
White		17 (77.3)		118 (83.7)		31 (86.1)	
Black		4 (18.2)		19 (13.5)		4 (11.1)	
Other		1 (4.5)		4 (2.8)		1 (2.8)	
BMI (kg/m^2)	22	27.1 ± 6.9	142	26.0 ± 5.1[3]	36	29.1 ± 5.9[2]	0.012[a]
Achalasia sub-type	14		120		34		0.023[d]
Subtype 1		5 (35.7)		30 (25.0)		13 (38.2)	
Subtype 2		6 (42.9)		82 (68.3)		18 (52.9)	
Subtype 3		2 (14.3)		1 (0.83)		3 (8.8)	
Achalasia variant		1 (7.1)		7 (5.8)		0 (0.0)	
Prior treatments							
Received any prior treatment	22	15 (68.2)	140	62 (44.3)[3]	36	26 (72.2)[2]	0.003[c]
Months from last to current treatment	14	17.7 [2.3, 87.5]	54	6.3 [3.2,28.5]	25	14.7 [6.4,20.2]	0.29[b]
Botulinium toxin injection	22	1 (4.5)	140	16 (11.4)	36	8 (22.2)	0.11[c]
PD	22	9 (40.9)[2]	140	19 (13.6)[1,3]	36	11 (30.6)[2]	0.002[c]
LHM	22	7 (31.8)[2]	140	1 (0.71)[1,3]	36	10 (27.8)[2]	< 0.001[c]
Botulinum toxin injection and regular endoscopic balloon dilation	22	2 (9.1)	140	1 (0.71)	36	0 (0.0)	0.050[d]
Regular endoscopic balloon dilation	22	3 (13.6)	140	34 (24.3)[3]	36	2 (5.6)[2]	0.031[c]

[1]Significantly different from PD; [2]Significantly different from LHM; [3]Significantly different from POEM. Values presented as Mean ± SD, Median [P25, P75] or N (column %). *P*-value: a = ANOVA, b = Kruskal-Wallis test, c = Pearson's χ^2 test, d = Fisher's Exact test. PD: Pneumatic dilation; LHM: Laparoscopic Heller myotomy; POEM: Peroral endoscopic myotomy.

as the independent variables. No adjustments were done for type of achalasia because (1) it was missing for > 15% of patients and (2) it is a 5 level variable. All analyses were performed using SAS version 9.4 (The SAS Institute, Cary, NC, United States) and a *P*-value < 0.05 was considered statistically significant. The statistical methods of this study were reviewed by Rocio Lopez, MS, Biostatistician from Department of Biostatistics, Cleveland Clinic, Cleveland, OH, United States.

RESULTS

A total of 200 achalasia patients were included of which 36 underwent POEM, 22 underwent PD and 142 underwent LHM. Baseline patient characteristics are summarized in Table 1. Patients who underwent POEM were significantly older compared to LHM patients (55.4 years *vs* 46.5 years, *P* = 0.013). POEM patients also had higher BMI compared to LHM patients (29.1 kg/m^2 *vs* 26 kg/m^2, *P* = 0.012). PD and POEM patients have had more prior treatments performed compared to LHM patients (68%, 72% and 44%, *P* = 0.003).

Pre-treatment and 2-mo post-treatment TBE and HREM findings in the three treatment groups are summarized in Tables 2 and 3. There was no significant difference in pre-treatment TBE and HREM parameters in all three treatments groups (*P* > 0.05). Post-treatment, there was significant improvement in TBE and HREM parameters in all three treatment groups. Both basal LES and LES-IRP pressures improved significantly after both POEM and LHM (*P* < 0.05). HREM was not routinely performed in all PD patients post-treatment and hence that data is not available. Actual LES-IRP at 2 mo decreased to less than 10 mmHg in 66/92 patients (71.7%) in LHM group, 19/26 patients (73.1%) in POEM group and 0/3 patients (0%) in PD group (*P* not significant). TBE parameters such as barium column height, width and volume remaining at both 1 min and 5 min improved significantly in all the three treatment groups (*P* < 0.05) except column height at 1 min in TBE group (*P* = 0.11). Actual barium column height at 5 min on TBE at 2 mo decreased by more than 50% in 73/131 patients (55.7%) in LHM group, 16/34 patients (47.1%) in POEM group and 7/20 patients (35%) in PD group (*P* not significant). Eckardt symptom scores improved significantly in both POEM and LHM patients (although only 7 patients had these available both pre- and post-treatment in LHM group). Eckardt scores were not available in PD group.

Details of multivariate analysis assessing pre- and post-treatment differences in HREM and TBE parameters in all three treatment groups are shown in Table 4. The degree of improvement in TBE parameters did not significantly differ among the three treatment groups (*P* > 0.05). Similarly, there was no significant difference in improvement in HREM parameters between the POEM and LHM groups (*P* > 0.05). Only 3 patients in the PD group had HREM testing done pre- and post-treatment, hence this group was not included

Table 2 High-resolution esophageal manometry and timed barium swallow findings: Univariable analysis

Factor	PD (*n* = 22)		LHM (*n* = 142)		POEM (*n* = 36)		*P* value
	n	Summary	*n*	Summary	*n*	Summary	
Pre-treatment							
Eckardt score	2	7.0 (7.0, 7.0)	9	6.0 (5.0, 7.0)	36	6.5 (5.0, 8.0)	0.77
HREM							
Basal mean pressure (mmHg)	2	31.9 (10.6, 53.2)	86	40.5 (27.2, 51.7)	24	38.7 (27.0, 48.7)	0.89
LES-IRP pressure (mmHg)	3	29.1 (12.0, 34.5)	92	24.0 (17.5, 34.4)	26	23.6 (20.2, 33.4)	0.92
TBE							
Height in 1 min (cm)	22	10.2 (7.0, 13.6)	133	9.5 (7.2, 15.0)	34	9.8 (4.0, 14.5)	0.43
Width in 1 min (cm)	22	3.4 (2.5, 4.0)	133	3.0 (2.5, 4.0)	34	3.4 (2.0, 4.4)	0.93
Volume remaining at 1 min (cc)	22	67.3 (44.0, 126.2)	133	71.6 (41.1, 131.9)	34	52.8 (37.7, 119.2)	0.44
Height in 5 min (cm)	20	6.5 (4.0, 10.5)	131	8.0 (5.0, 12.5)	34	5.3 (2.5, 10.0)	0.063
Width at 5 min (cm)	20	2.7 (2.0, 3.6)	131	2.5 (2.0, 3.7)	34	2.5 (1.5, 4.0)	0.83
Volume remaining at 5 min (cc)	20	40.8 (15.5, 73.1)	131	49.1 (15.7, 91.6)	34	25.4 (11.3, 62.8)	0.12
2-mo post-treatment							
Eckardt score	4	4.5 (2.0, 6.0)	50	1.00 (0.00, 2.0)	36	1.00 (0.00, 2.0)	0.073
HREM							
Basal mean pressure (mmHg)	2	22.0 (18.8, 25.1)	86	14.5 (7.6, 22.7)	24	11.4 (8.2, 20.2)	0.32
LES-IRP pressure (mmHg)	3	10.8 (10.5, 19.4)	92	7.1 (3.9, 10.7)	26	6.6 (3.3, 11.1)	0.18
TBE							
Height in 1 min (cm)	22	8.0 (5.8, 11.0)	133	6.7 (4.5, 10.2)	34	6.3 (2.1, 9.5)	0.39
Width in 1 min (cm)	22	2.0 (1.5, 2.5)	133	2.0 (1.2, 2.5)	34	1.6 (0.50, 2.5)	0.28
Volume remaining at 1 min (cc)	22	25.4 (14.1, 41.7)	133	20.4 (6.0, 49.8)	34	12.8 (0.79, 47.7)	0.31
Height in 5 min (cm)	20	2.2 (0.00, 6.5)	131	2.5 (0.00, 6.2)	34	2.3 (0.00, 6.9)	0.94
Width at 5 min (cm)	20	1.05 (0.00, 2.6)	131	1.00 (0.00, 2.1)	34	0.50 (0.00, 2.0)	0.97
Volume remaining at 5 min (cc)	20	4.1 (0.00, 30.2)	131	2.7 (0.00, 21.2)	34	0.54 (0.00, 18.8)	0.98
Post - pre treatment difference							
Eckardt score	-	-	7	-6.0 (-8.0, -2.0)	36	-6.0 (-7.0, -4.0)	0.75
HREM							
Post-Pre basal mean pressure (mmHg)	2	-10.0 (-34.4, 14.5)	86	-25.1 (-36.8, -12.1)	24	-19.6 (-43.1, -11.9)	0.78
Post-Pre LES-IRP pressure (mmHg)	3	-9.7 (-23.7, -1.5)	92	-15.2 (-26.4, -8.3)	26	-14.2 (-24.5, -7.8)	0.60
TBE							
Post-Pre height at 1 min (cm)	22	-0.90 (-5.5, 1.9)	133	-2.5 (-7.0, 0.30)	34	-2.8 (-8.5, 1.5)	0.73
Post-Pre width at 1 min (cm)	22	-1.4 (-2.0, -0.30)	133	-1.00 (-2.0, -0.20)	34	-1.5 (-2.1, 0.00)	0.79
Post-Pre volume at 1 min (cc)	22	-40.2 (-81.2, -14.1)	133	-35.8 (-101.8, -10.4)	34	-29.5 (-100.7, -0.29)	0.74
Post-Pre height at 5 min (cm)	20	-0.75 (-5.4, 0.05)	131	-4.7 (-10.0, 0.00)	34	-2.0 (-7.5, 1.9)	0.069
Post-Pre width at 5 min (cm)	20	-0.90 (-2.3, 0.00)	131	-1.5 (-2.2, -0.20)	34	-1.00 (-2.4, 0.00)	0.86
Post-Pre volume at 5 min (cc)	20	-14.0 (-45.9, -2.6)	131	-31.3 (-66.1, -5.5)	34	-17.0 (-37.7, 0.00)	0.14

Values presented as Median (P25, P75) with Kruskal-Wallis tests. PD: Pneumatic dilation; LHM: Laparoscopic Heller myotomy: POEM: Peroral endoscopic myotomy; HREM: High resolution esophageal manometry; TBE: Timed barium esophagram; LES: Lower esophageal sphincter; IRP: Integrated relaxation pressure.

in the multivariate analysis on HREM.

DISCUSSION

Our study showed that all three treatment modalities for achalasia namely PD, LHM and POEM were effective in improving esophageal function evaluated at 2 mo post-treatment. All three treatments resulted in significant improvement in esophageal emptying on TBE. Both POEM and LHM led to significant decrease in LES pressures on HREM. More importantly, this is the first study that demonstrates efficacy of all three treatments and that there was no significant difference in efficacy between the three treatments on short term follow-up.

Pre and post-treatment physiologic evaluation of esophageal function in achalasia by HREM is very important to assess the improvement after treatment and also to predict long term response. HREM parameters such as LES-IRP were shown to correlate with symptom scores of achalasia[17,18]. Several studies in achalasia patients treated with PD and LHM have shown that the HREM parameters also predict long term need for retreatment[6-9,19]. As such LES-IRP of greater than 10 mmHg after treatment was predictive of requiring retreatment on follow-up. In our study, LES-IRP decreased significantly after treatment in all three treatment modalities (although only 3 patients in PD group had HREM post-treatment). Post-treatment LES-IRP was only 7.1 mmHg and 6.6 mmHg in LHM and POEM groups respectively, and hence we predict our patients would have excellent long term efficacy. Teitelbaum *et al*[12] have shown that decreased LES-IRP at 2 mo after POEM persisted at 1 year as well, which supports our long-term prediction in our POEM and LHM groups.

Table 3 Improvement in high resolution esophageal manomtery and timed barium esophagram parameters in each treatment group

Factor	*n*	PD (*n* = 22) Pre-Treatment	Post-treatment	*P* value
HREM[1]				
TBE				
Height at 1 min (cm)	22	10.2 (7.0, 13.6)	8.0 (5.8, 11.0)	0.11
Width at 1 min (cm)	22	3.4 (2.5, 4.0)	2.0 (1.5, 2.5)	< 0.001
Volume at 1 min (cc)	22	67.3 (44.0, 126.2)	25.4 (14.1, 41.7)	< 0.001
Height at 5 min (cm)	20	6.5 (4.0, 10.5)	2.2 (0.00, 6.5)	0.026
Width at 5 min (cm)	20	2.7 (2.0, 3.6)	1.05 (0.00, 2.6)	< 0.001
Volume at 5 min (cc)	20	40.8 (15.5, 73.1)	4.1 (0.00, 30.2)	0.001
		LHM (*n* = 142)		
Factor				
HREM				
Basal mean pressure (mmHg)	86	40.5 (27.2, 51.7)	14.5 (7.6, 22.7)	< 0.001
LES-IRP pressure (mmHg)	92	24.0 (17.5, 34.4)	7.1 (3.9, 10.7)	< 0.001
TBE				
Height at 1 min (cm)	133	9.5 (7.2, 15.0)	6.7 (4.5, 10.2)	< 0.001
Width at 1 min (cm)	133	3.0 (2.5, 4.0)	2.0 (1.2, 2.5)	< 0.001
Volume at 1 min (cc)	133	71.6 (41.1, 131.9)	20.4 (6.0, 49.8)	< 0.001
Height at 5 min (cm)	131	8.0 (5.0, 12.5)	2.5 (0.00, 6.2)	< 0.001
Width at 5 min (cm)	131	2.5 (2.0, 3.7)	1.00 (0.00, 2.1)	< 0.001
Volume at 5 min (cc)	131	49.1 (15.7, 91.6)	2.7 (0.00, 21.2)	< 0.001
		POEM (*n* = 36)		
Factor				
Eckardt score	36	6.5 (5.0, 8.0)	1.00 (0.00, 2.0)	< 0.001
HREM				
Basal mean pressure (mmHg)	24	38.7 (27.0, 48.7)	11.4 (8.2, 20.2)	< 0.001
LES-IRP pressure (mmHg)	26	23.6 (20.2, 33.4)	6.6 (3.3, 11.1)	< 0.001
TBE				
Height at 1 min (cm)	34	9.8 (4.0, 14.5)	6.3 (2.1, 9.5)	0.01
Width at 1 min (cm)	34	3.4 (2.0, 4.4)	1.6 (0.50, 2.5)	< 0.001
Volume at 1 min (cc)	34	52.8 (37.7, 119.2)	12.8 (0.79, 47.7)	< 0.001
Height at 5 min (cm)	34	5.3 (2.5, 10.0)	2.3 (0.00, 6.9)	0.017
Width at 5 min (cm)	34	2.5 (1.5, 4.0)	0.50 (0.00, 2.0)	< 0.001
Volume at 5 min (cc)	34	25.4 (11.3, 62.8)	0.54 (0.00, 18.8)	0.003

[1]HREM data not available in PD group. Values presented as Median (P25, P75) with Wilcoxon signed rank test. PD: Pneumatic dilation; LHM: Laparoscopic Heller myotomy; POEM: Peroral endoscopic myotomy; HREM: High resolution esophageal manometry; TBE: Timed barium esophagram; LES: Lower esophageal sphincter; IRP: Integrated relaxation pressure.

Esophageal emptying assessed by a TBE is a complementary test to HREM for functional assessment of esophageal physiology. Similar to LES-IRP, post-treatment improvement in esophageal emptying is a predictor of the need for retreatment in achalasia[3,10]. Vaezi *et al*[10] have shown that successful esophageal emptying, defined as at least 50% reduction of barium column after treatment, was associated with long-term remission of symptoms. In that study, patients with sub-optimal esophageal emptying after PD required retreatments on long-term follow-up. In our study, barium column height decreased by more than 50% in all three treatments groups at 2 mo follow-up, reinforcing the efficacy of all three treatments. In our POEM patients, Eckardt scores improved significantly paralleling the improvement in LES pressures. However, we suspect to have had similar decrease in Eckardt scores in LHM and PD groups if they were available, since LES pressures decreased significantly in those patients as well. There was also no significant difference in esophageal emptying between the three treatment groups, reinforcing comparable efficacy of all three treatment modalities.

In our study, there were some notable differences in patient characteristics among PD, LHM and POEM groups. Patients in POEM and PD treatments groups were older, had higher BMI, and more likely to have received prior treatments. This is likely due to the selection bias of a particular treatment modality for different patients at our institution. Usually younger patients and fit surgical candidates were offered LHM due to its well established long term durability record. Older and somewhat less ideal surgical candidates were preferentially offered either PD or POEM. Initially the following subsets of patients were considered for POEM: (1) Obese patients, patients with upper abdominal surgical scars *i.e.*, hostile abdomen and those with prior failed LHM, in whom LHM is technically difficult or less desirable; and (2) patients over 60 years of age (not younger patients since long term cumulative effects of gastroesophageal reflux disease (GERD) after POEM are not yet known). However, we do not believe that this selection bias should have affected the results of our study significantly.

Table 4 High resolution esophageal manomtery and Timed barium esophagram findings: Adjusted analysis[1]

Outcome	PD	LHM	POEM	*P* value
Eckardt score	[2]	-5.7 (-6.7, -4.5)	-5.6 (-6.1, -5.2)	0.94
HREM				
Post-Pre basal mean pressure (mm Hg)	[2]	-27.5 (-30.3, -24.5)	-33.1 (-38.0, -27.6)	0.084
Post-Pre LES-IRP pressure (mm Hg)	[2]	-20.1 (-22.2, -17.9)	-20.9 (-24.7, -16.5)	0.76
TBE				
Post-Pre height at 1 min (cm)	-2.7 (-5.1, -0.05)	-4.6 (-5.5, -3.7)	-5.4 (-7.1, -3.5)	0.21
Post-Pre width at 1 min (cm)	-1.4 (-1.9, -0.77)	-1.3 (-1.5, -1.03)	-1.7 (-2.2, -1.2)	0.28
Post-Pre volume at 1 min (cc)	-81.8 (-112.4, -44.3)	-79.3 (-92.7, -64.8)	-95.6 (-119.4, -67.6)	0.58
Post-Pre height at 5 min (cm)	-5.8 (-7.7, -3.7)	-6.0 (-6.8, -5.2)	-5.8 (-7.3, -4.1)	0.95
Post-Pre width at 5 min (cm)	-1.6 (-2.4, -0.74)	-1.5 (-1.8, -1.1)	-1.9 (-2.5, -1.3)	0.47
Post-Pre volume at 5 min (cc)	-68.2 (-89.9, -43.5)	-51.2 (-60.9, -41.0)	-64.6 (-82.0, -45.3)	0.28

[1]ANOVA analysis was used to obtain adjusted means. A logarithm transformation of each outcome [ln(y -1 + min(y)] was modeled as the outcome variable with age at time of treatment, BMI and having had previous treatments as the independent variables. 2Data not available Only 3 patients in PD group had HREM testing done both pre- and post-treatment, hence this group was not included in the models. Values presented as mean (95%CI). PD: Pneumatic dilation; LHM: Laparoscopic Heller myotomy; POEM: Peroral endoscopic myotomy; HREM: High resolution esophageal manomtery; TBE: Timed barium esophagram; LES: Lower esophageal sphincter; IRP: Integrated relaxation pressure.

There are some limitations in our study including its retrospective design and only short-term follow-up. The details of patients' symptoms such as Eckardt scores were not available in all our patients except in the POEM group. Only 3/22 patients had HREM after treatment in the PD group. Details about GERD, a common adverse effect of any achalasia treatment, were not available and hence were not included in this study. It is also beyond the scope of this paper and we acknowledge it as one of the limitations of our study. Evaluation of esophagogastric junction (EGJ) distensibility by EndoFlip is another parameter being used for assessing esophageal physiology and is a useful predictor of treatment outcomes[6]. EGJ distensibility was however, not assessed in our patients. The main strength of our study lies in the real world scenario of treating patients with established achalasia and a large number of patients in the study. All patients had multi-disciplinary clinical evaluation by gastroenterologists, thoracic surgeons and radiologists, along with TBE and HREM before and after treatment. This is also the first study which compared the efficacy of all three standard treatments of achalasia in a large number of patients.

In conclusion, this study shows that all three treatments of achalasia namely POEM, LHM and PD lead to improvement in esophageal function as assessed by HREM and TBE in the short-term. These results support the selection of any of the three treatment modalities based on patient characteristics and availability of local expertise to perform these procedures. Larger, prospective studies with homogeneous patient populations and longer follow-up are required to compare the efficacy of these treatment modalities in achalasia.

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COMMENTS

Background

Achalasia is a primary esophageal motility disorder characterized by esophageal aperistalsis and impaired relaxation of lower esophageal sphincter. Standard treatments are palliative and include laparoscopic Heller myotomy (HLM) and endoscopic pneumatic dilation (PD). Recently peroral endoscopic myotomy is rapidly emerging as a standard treatment as well. This study evaluated and compared the efficacy of peroral endoscopic myotomy *vs* other standard treatments of achalasia in improving esophageal function.

Research frontiers

Peroral endoscopic myotomy is gaining popularity due to its minimal invasiveness of an endoscopic procedure and high precision of a surgical myotomy. There are several studies comparing peroral endoscopic myotomy with either PD or HLM. This study compared the efficacy of all three treatment modalities in improving esophageal function. The study findings help the peers in appropriate selection of each treatment modality based on local expertise and availability.

Innovations and breakthroughs

Recent innovations in the achalasia include emergence of peroral endoscopic myotomy as a standard treatment modality. Several studies have shown its effectives in palliation of symptoms comparable to other treatments such as PD and HLM. This study evaluated and compared the efficacy of all three standard treatments in improving esophageal function objectively by timed barium esophagram and high resolution esophageal manometry. Peroral endoscopic myotomy was effective and was comparable to other treatments in improving esophageal function in the short term in patients with achalasia.

Applications

This study results suggested that perooral endoscopic myotomy is effective not only in proving symptoms but also objective esophageal function in achalasia similar to PD and HLM. Furthermore, the study findings have practical implications in the sense that selection of one of the three treatment modalities may be done based on local expertise and patient choice.

Terminology

Achalasia is rare primary esophageal disorder characterized by esophageal peristasis and impaired relaxation of lower esophageal sphincter. Treatment of achalasia is aimed at palliation of symptoms by disruption of lower esophageal sphincter. Standard treatments include endoscopic PD, HLM and recently

emerging incisionless peroral endoscopic myotomy.

Peer-review

There is paucity of data comparing the efficacy of all three treatment modalities of achalasia namely HLM, PD and peroral endoscopic myotomy in improving objective esophageal function. This study showed that all three treatments modalities are effective and comparable in the short term. These findings have important practical implications in the treatment of patients with achalasia.

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ORIGINAL ARTICLE

Prospective Study

Acoustic radiation force impulse imaging for assessing liver fibrosis in alcoholic liver disease

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Clinical trial registration statement: This study is registered at https://clinicaltrials.gov/ct2/show/NCT01789008?term=NCT01789008&rank=1. The registration identification number is NCT01789008.

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Abstract

AIM: To evaluate the performance of elastography by ultrasound with acoustic radiation force impulse (ARFI) in determining fibrosis stage in patients with alcoholic liver disease (ALD) undergoing alcoholic detoxification in relation to biopsy.

METHODS: Eighty-three patients with ALD undergoing detoxification were prospectively enrolled. Each patient underwent ARFI imaging and a liver biopsy on

the same day. Fibrosis was staged according to the METAVIR scoring system. The median of 10 valid ARFI measurements was calculated for each patient.

RESULTS: Sixty-nine males and thirteen females (one patient excluded due to insufficient biopsy size) were assessed with a mean alcohol consumption of 132.4 ± 128.8 standard drinks per week and mean cumulative year duration of 17.6 ± 9.5 years. Sensitivity and specificity were respectively 82.4% (0.70-0.95) and 83.3% (0.73-0.94) (AUROC = 0.87) for F ⩾ 2 with a cut-off value of 1.63m/s; 82.4% (0.64-1.00) and 78.5% (0.69-0.89) (AUROC = 0.86) for F ⩾ 3 with a cut-off value of 1.84m/s; and 92.3% (0.78-1.00] and 81.6% (0.72-0.90) (AUROC = 0.89) for F = 4 with a cut-off value of 1.94 m/s.

CONCLUSION: ARFI is an accurate, non-invasive and easy method for assessing liver fibrosis in patients with ALD undergoing alcoholic detoxification.

Key words: Alcoholic liver disease; Elastography; Non-invasive; Acoustic radiation force impulse; Fibrosis

Core tip: The aim of this study was to evaluate the performance of elastography by ultrasound with acoustic radiation force impulse (ARFI) in determining fibrosis stage in patients with alcoholic liver disease (ALD) undergoing alcoholic detoxification. Compared to biopsy, ARFI is an accurate, non-invasive and easy method for assessing liver fibrosis in patients with ALD undergoing alcoholic detoxification, with a good sensitivity and specificity.

Kiani A, Brun V, Lainé F, Turlin B, Morcet J, Michalak S, Le Gruyer A, Legros L, Bardou-Jacquet E, Gandon Y, Moirand R. Acoustic radiation force impulse imaging for assessing liver fibrosis in alcoholic liver disease. *World J Gastroenterol* 2016; 22(20): 4926-4935 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i20/4926.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i20.4926

INTRODUCTION

Chronic alcohol abuse is a major public health problem worldwide. Alcoholic liver disease (ALD) is one of the most common complications and a leading cause of alcohol-related death, due to liver cirrhosis and its complications. In 2004, 3.8% of all global deaths and 4.6% of global disability-adjusted life-years were attributable to alcohol consumption. The treatment of alcoholic liver disease generates substantial costs for the healthcare system[1].

Three histological lesions characterise ALD: steatosis, steatohepatitis and fibrosis. There are different stages of fibrosis, the last of which is cirrhosis. In ALD, it is important to know the fibrosis stage in order to guide management decisions and estimate prognosis. The appropriate intervention strategies can prevent serious long-term outcomes[2]. Patients with cirrhosis run a greater risk of complications (portal hypertension, hepatocellular carcinoma, ascites, *etc.*) and need closer follow-up. Informing patients of their cirrhosis could also provide an incentive to stop alcohol consumption. However, patients with severe fibrosis or cirrhosis are completely clinically asymptomatic for a long period of time and can be difficult to diagnose.

Liver biopsy is the gold standard for assessment of liver fibrosis, evaluating fibrosis, steatosis and the necroinflammatory stage at the same time. However, biopsy is an invasive procedure associated with morbidity and minor complications (local discomfort at the biopsy site, pain and transient hypotension due to a vasovagal reaction) reported in 5%-20% of cases and major complications (bleeding and peritonitis) in 0.3%-0.6% of cases. The mortality rate is 0.01%-0.3%[3]. Moreover, there are other considerations such as contraindications (ascites, intrahepatic biliary duct dilation, coagulation disorders), insufficient sampling and inter-observer variability. A 1-d hospital stay is also necessary, leading to significant costs.

This led to the development of alternative non-invasive methods for assessing hepatic fibrosis in alcoholic liver disease. Serum markers alone or in combination with specific algorithms have been used for the non-invasive assessment of liver fibrosis. Examples include Fibrotest®, Forns index and APRI[4-6]. The limitations of these tests are the influence of comorbid conditions and a lack of liver specificity. Another method involves measuring the elasticity of liver tissue (liver stiffness) which is markedly influenced by the stage of fibrosis. The most popular method for measuring liver stiffness is transient elastography (TE) by Fibroscan®, which has been validated for hepatitis C liver disease patients. Some authors have even combined TE and serum markers[7].

A new ultrasound technique has recently emerged: acoustic radiation force impulse (ARFI) elastography. ARFI could be of great utility in the measurement of liver fibrosis in alcoholic liver disease. This non-invasive method has the particular advantage of combining conventional ultrasound and liver stiffness measurement. Ultrasound is the primary imaging technique used worldwide to evaluate diffuse hepatic diseases. Acoustic radiation force is a phenomenon associated with the propagation of acoustic waves in attenuating media. The device generates a short-duration (262 ms) acoustic pulse by ultrasound. This pulse creates mechanical excitation and displacement of tissue. The deformation induced by the acoustic pulse is followed by a relaxation process after which the tissue returns to its original configuration, generating a shear wave. The speed of this wave is cal-

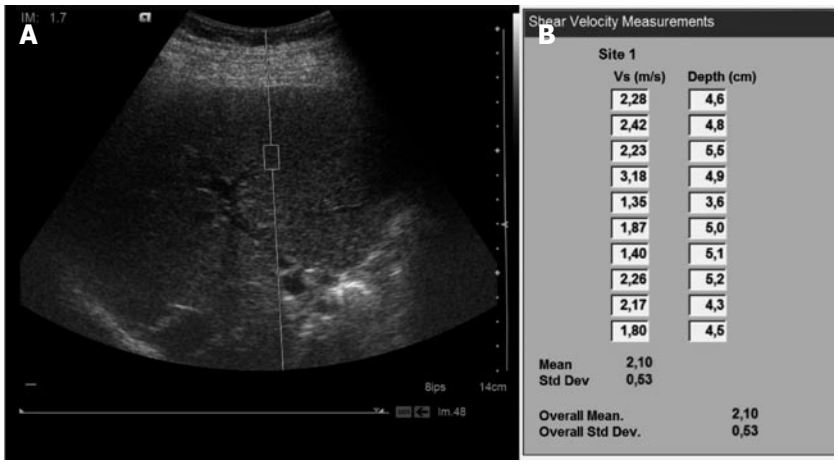

Figure 1 Image of liver stiffness measurement by acoustic radiation force impulse in patients with alcoholic liver disease. A: Acoustic radiation force impulse examination of a patient; B: Ten values with associated depths.

culated, providing a quantitative measurement. The shear wave speed of the tissue can be reconstructed as soft tissues are elastic and deformed more easily than rigid tissue.

In the past few years, ARFI has started to be assessed in comparison to biopsy, TE and biological markers. These studies mainly involved hepatitis B, hepatitis C and non-alcoholic steatohepatitis (NASH).

The aim of our prospective study was to evaluate the performance of ARFI in determining fibrosis stage in patients with alcoholic liver disease in relation to biopsy.

MATERIALS AND METHODS

Subjects

The local ethics committee approved this study. All patients gave written informed consent prior to enrolment. This study is an ancillary single-centre study of a larger, ongoing, multi-centre trial on validation of non-invasive fibrosis tests in ALD. Clinical Trials Identifier: NCT01789008.

From February 2013 to June 2015, the study was offered to all patients referred to the University Hospital of Rennes, France, who were admitted to the addiction treatment unit in the liver disease department for detoxification with an indication of liver biopsy for alcoholic liver disease. The inclusion criteria were: patients over 18 years old, hospitalisation for alcoholic detoxification, high-risk alcohol consumption (more than 210 g of alcohol per week for men and 140 g of alcohol per week for women) for a cumulative period of more than 5 years, a rise in serum aspartate transferase (AST) greater than 1.5 the upper limit of the normal range, associated with a rise in gamma-glutamyl transferase (GGT) and not explained by another cause of liver disease. When patients had features of metabolic syndrome or were obese, liver disease was felt to be principally due to alcohol consumption when the AST/alanine amino transferase (ALT) ratio was greater than one, and GGT was markedly high, and when the abnormalities decreased with alcohol withdrawal. Non-inclusion criteria were: cirrhosis already known or obvious due to clinical and biological signs (ascites, increased prothrombin time or oesophageal varices), other causes of hepatic disease (viral, autoimmune or cholestatic disease) or contraindication to biopsy. The interval between alcohol cessation and the procedure was not more than 10 d. Eighty-three patients were prospectively enrolled.

ARFI elastography

ARFI imaging was performed with a Siemens Acuson S2000TM ultrasound system (Siemens AG, Erlangen, Germany), with software version VB10D and a 4C1 curved ultrasound probe. The region of interest (ROI) of 10 mm length and 5 mm width was placed while performing B-mode imaging in the right lobe of the liver at a maximum depth of 8 cm, avoiding large vessels, biliary ducts and potential lesions (Figure 1). The operator applied the minimum pressure required to take the image.

Patients were in fasted state. None had cardiac disease. They were in the supine position with the right arm in maximum abduction and were asked to stop normal breathing for a moment and not inhale or exhale deeply. The aim was to minimise breathing motion and avoid inhaling/exhaling, which are known influencing factors[8]. The probe was placed between and parallel to the seventh to tenth intercostal space.

Ten valid acquisitions were obtained for each patient, in the same intercostal space but with different locations and at different depths. Each acquisition period was about 10-15 s long. The median of all 10 acquisitions was calculated and considered as indicative of fibrosis severity. The results were expressed in m/s. For each measurement, the depth of the box was given. Measurements were obtained at a depth of 1 cm from the liver capsule down to a maximum depth of 8 cm below the transducer. If the measurement was

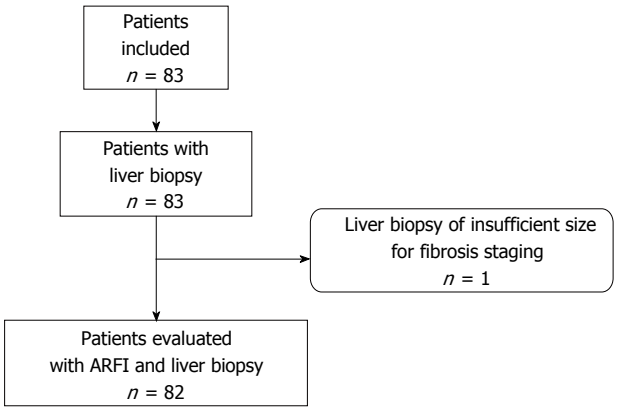

Figure 2 Patient flowchart. ARFI: Acoustic radiation force impulse.

technically evaluated as non-reliable by the device, X.XX was displayed on the screen. Reliable, successful liver stiffness measurements were defined as the median of 10 valid measurements with a success rate ⩾ 60% (based on TE).

The operator was blinded for all patient characteristics including clinical, biological and histological data.

Abdominal ultrasound

Liver and abdominal ultrasound imaging was performed at the same time for all patients using the same device and probe as for the ARFI examination. We recorded the right liver lobe size (right liver arrow), the distance between the skin and the superficial liver capsule, the liver structure and any focal liver lesion.

Liver biopsy

Liver biopsy was performed under percutaneous ultrasound guidance after ARFI acquisition on the same day. The liver samples were fixed and for each patient three slides were stained with hematoxylin-eosin and Sirius red. To avoid sampling errors, specimens under 15 mm long were excluded. A senior pathologist, blinded to clinical, histological, biological and ARFI data, assessed the liver biopsies according to the METAVIR scoring system[9]. Fibrosis was staged from 0 to 4 determined according to the METAVIR score: F = fibrosis. F0: no fibrosis; F1: portal fibrosis without septa (minimal fibrosis); F2: portal fibrosis with rare septa (moderate fibrosis); F3: numerous septa without cirrhosis (severe fibrosis); and F4: cirrhosis. Perisinusoidal fibrosis was evaluated according to Brunt's score[10].

Clinical and biological parameters

Biological parameters were measured prior to liver biopsy and ARFI. These included: prothrombin, alkaline phosphatase, albumin, γ-globulin, platelets, AST, ALT, γ-glutamyl transferase, iron and ferritin. Other parameters were age, sex, body mass index (BMI) and hypertension.

Statistical analysis

Groups of patients were formed according to fibrosis stage and data were expressed as mean ± SD if normally distributed and median (range) if not normally distributed. Comparisons between groups were made using *t*-tests for normally distributed variables, Mann Whitney *U* test for non-normally distributed variables and the χ^2 test or Fisher's exact test for categorical variables. Spearman's analysis was used to determine any correlations. Optimal cut-off values for fibrosis stages F ⩾ 2, F ⩾ 3 and F = 4 were determined by optimisation of Youden's index from the AUROC curve analysis. Sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV) were also calculated. The variables tested in the univariate analysis were ARFI, effects of prothrombin, alkaline phosphatase, albumin, γ-globulin, platelets, AST, ALT, γ-glutamyl transferase, iron, ferritin, age, sex, BMI and hypertension ($P < 0.2$). Multivariate ordinal logistic regression analysis using fibrosis stage (in three classes: F0-F1/F2/F3-F4) as the outcome variable was used to assess the strength of the relationship with ARFI even after adjustments for other factors associated with fibrosis progression or confusion factors. A P value of < 0.05 was considered statistically significant. An algorithm was developed using the clustering method. Statistical analyses were performed using SAS V9.4 software (SAS Institute, United States).

RESULTS

Eighty-two patients (69 males and 13 females) were evaluated in the analysis. One enrolled patient was excluded due to insufficient biopsy size (Figure 2). The mean age of the patients was 43.8 ± 10 years and the mean BMI was 22.9 ± 4.3 kg/m^2. Mean alcohol consumption was 132.4 ± 128.8 standard drinks (defined as 10 g of pure alcohol per standard drink in France) per week with a mean cumulative year duration of 17.6 ± 9.5 years. Mean biopsy size was 30.7 ± 10.5 mm. Patient characteristics and fibrosis stages are summarised in Tables 1 and 2 respectively. Successful liver stiffness measurements (10 valid measurements) were obtained in 100% of patients measured with ARFI imaging.

The median values for ARFI imaging according to fibrosis stage are described in Table 2 (mean of medians ± SD). The results showed a significant and strong correlation between ARFI measurements and the histological fibrosis stage ($P < 0.0001$) (Figure 3). Analyses to determine the optimal ARFI cut-off values were performed according to stages of clinical interest, for F ⩾ 2, ⩾ F3 and F = 4. AUROC values were respectively 0.87, 0.86 and 0.89 (Figure 4). Sensitivity, specificity, PPV and NPV are shown in Table 3 according to the cut-off values.

Table 1 Clinical and biochemical characteristics of patients with alcoholic liver disease

Characteristic	Normal values	Patients included (n = 82)
Sex (male/female)	NA	69/13
Age (yr)	NA	43.8 ± 10
Body mass index (kg/m^2)	NA	22.9 ± 4.3
AST (IU/L)	0-35	62.0 (44-98)
ALT (IU/L)	0-35	67.0 (40-105)
γ-glutamyl transpeptidase (IU/L)	5-36	316.0 (141-654)
γ-globulin (g/L)	7-15	8.7 (7.4-10.4)
Alkaline phosphatase (IU/L)	30-120	90.5 (66-121)
Prothrombin (%)	70-130	101.2 ± 12.4
Platelets (× 10^9/L)	180-390	191.5 ± 71.7
Iron (μmol/L)	18-22	16.2 ± 8.1
Ferritin (μg/L)	Male: 30-300	478.0 (310.5-787.5)
	Female: 20-150	404.0 (216.0-783.0)
Albumin (g/L)	40-60	39.0 ± 4.9

Kolmogorow Smirnow analysis was performed, followed by parametric or non-parametric tests. Mean ± SD or median (25th-75th interquartile ranges) was used respectively. AST: Aspartate transaminase; ALT: Alanine transaminase; IU: International unit.

Table 2 Number of patients and mean values of acoustic radiation force impulsepredicting for assessing liver stiffness according to the different fibrosis stages

Fibrosis stage	Number of patients	ARFI (m/s)
F0	13	1.25 ± 0.31
F1	35	1.40 ± 0.36
F2	17	1.86 ± 0.42
F3	4	1.83 ± 0.47
F4	13	2.25 ± 0.36
P value		< 0.0001

ARFI: Acoustic radiation force impulsepredicting.

Significant variables in univariate analyses (P < 0.2) as described previously were entered into the multivariate model (Table 4). The proportional odds assumption was verified (P = 0.76) and the coefficient of determination (R²) was 61%. Age ⩾ 50 [OR = 4.73 (1.43-15.66)] and γ-globulin ⩾ 10 g/L [OR = 9.67 (2.19-42.63)] were independently associated with fibrosis stage. Moreover, the relationship between fibrosis and ARFI was still significant [40.71 (9.94-166.7)] after adjusting for those additional parameters.

The correlation between the mean and median of the 10 values was excellent with a Spearman correlation coefficient of 0.98 (P < 0.001).

Medians were calculated using a different number of values (2-9) to determine whether or not there was a need for 10 values. The values were taken in order. Spearman's correlation coefficient is 0.98 between the median of 10 and 6 values (P < 0.0001).

DISCUSSION

This study demonstrates that ARFI imaging could be used for the assessment of liver fibrosis in ALD. We suggest that a median of 1.63 m/s could be used as an ARFI diagnostic threshold for diagnosing significant liver fibrosis (F ⩾ 2) with sensitivity and specificity of respectively 82.4% and 83.3% (AUROC = 0.87). Moreover, the threshold of 1.94 m/s provided a diagnosis of cirrhosis with a sensitivity of 92.3% and specificity of 81.6%.

Table 3 Diagnostic performance of acoustic radiation force impulse for the different liver fibrosis stages

Diagnostic parameters	F ⩾ 2	F ⩾ 3	F = 4
ARFI cut-off (m/s)	1.63	1.84	1.94
Sensitivity (%)	82.4 (0.70-0.95)	82.4 (0.64-1.00)	92.3 (0.78-1.00)
Specificity (%)	83.3 (0.73-0.94)	78.5 (0.69-0.89)	81.6 (0.72-0.90)
Area under curve (AUROC)	0.87	0.86	0.89
PPV (%)	77.8 (0.64-0.91)	50.0 (0.31-0.69)	48.0 (0.28-0.68)
NPV (%)	87.0 (0.77-0.97)	94.4 (0.88-1.00)	98.2 (0.95-1.00)

ARFI: Acoustic radiation force impulse; PPV: Positive predictive value; NPV: Negative predictive value.

Table 4 Univariate and multivariate ordinal regression analyses

Characteristic	Univariate analysis OR (95%) P value	Multivariate analysis OR (95%) R^2 = 0.61; STPOA[1]: 0.76
ARFI	29.06 [8.59-98.33] P < 0.0001	40.71 [9.94-166.7] P < 0.0001
Sex		
Female	1	
Male	2.43 [0.79-7.42] P = 0.12	
Age		
< 50 yr	1	1
⩾ 50 yr	4.01 [1.55-10.39] P = 0.004	4.73 [1.43-15.66] P = 0.01
Body mass index	1.07 [0.97-1.18] P = 0.19	
AST	1.00 [0.992-1.01] P = 0.98	
ALT	0.99 [0.98-0.999] P = 0.04	
γ-glutamyl transpeptidase	1.00 [1.000-1.002] P = 0.05	
γ-globulin		
< 10	1	1
⩾ 10	7.42 [2.34-23.56] P = 0.003	9.67 [2.19-42.63] P = 0.004
Alkaline phosphatase	1.01 [1.001-1.012] P = 0.02	
Prothrombin	0.002 [0.001-0.06] P = 0.001	
Platelets	1.00 [0.99-1.01] P = 0.93	
Iron	1.03 [0.97-1.08] P = 0.33	
Ferritin	1.001 [1.000-1.002] P = 0.06	
Albumin	0.90 [0.82-1.00] P = 0.05	

[1]Score test for the proportional odds assumption.

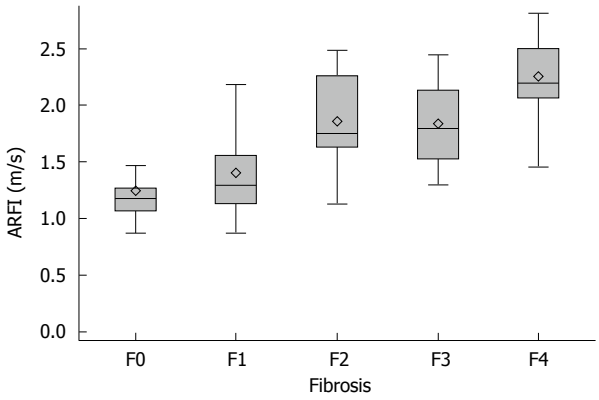

Figure 3 Relationship between acoustic radiation force impulse predicting values and histological fibrosis stages. The relationship was significant between the velocity of the sheer wave (median of 10 ARFI values) and the fibrosis stage (assessed by the METAVIR score on biopsies) in all 82 patients ($P < 0.0001$). The box represents the interquartile range, the horizontal line in the box indicates the median value and the diamond indicates the mean value. The horizontal lines above and below the box indicate the maximum and minimum values.

In alcoholic liver disease, the identification of cirrhosis (F4) is important for optimal patient care. The follow-up schedule includes endoscopy every 3-4 years, ultrasonography every 6 mo, hepatitis vaccination and contraindication to certain drugs. The identification of stage ⩾ F2 is less important clinically than in viral hepatitis but can lead to closer medical surveillance of fibrosis with ARFI, and even serve as an incentive for detoxification. For both stages, the AUROC curve value was close to 1, indicating good diagnostic accuracy[11].

To date, the predominant and most reliable non-invasive method for the diagnosis of liver fibrosis in alcoholic liver disease is transient elastography (TE)[12]. Compared to TE, ARFI has several advantages. First, B-mode evaluation of the liver (and other organs such as the spleen) is possible with the same device and can therefore be incorporated into routine ultrasound protocols, thereby reducing costs. The use of B-mode can also determine optimal ROI placement, preserving structures such as lesions, large blood vessels, biliary ducts or even heterogeneous areas. Second and probably most importantly is the liver stiffness measurement success rate of 100%, which was reported both in the literature and in our study, whereas in some studies TE has a success rate of under 70%[13,14]. This is a major strength of the ARFI method compared to TE. Third, ARFI imaging can be performed in some cases where TE is not possible[15]. Bota *et al*[16] demonstrated that the presence of ascites did not influence the ARFI measurement reliability rate, whereas TE cannot be performed in the case of ascites. TE is unreliable for overweight and obese patients whereas ARFI can be performed to a maximum depth of 8 cm. Published data also suggest that ARFI may not be influenced by steatosis grade, unlike TE[14,17,18]. This is a clear advantage in our population, as steatosis is often associated with ALD[19]. ARFI is also a good

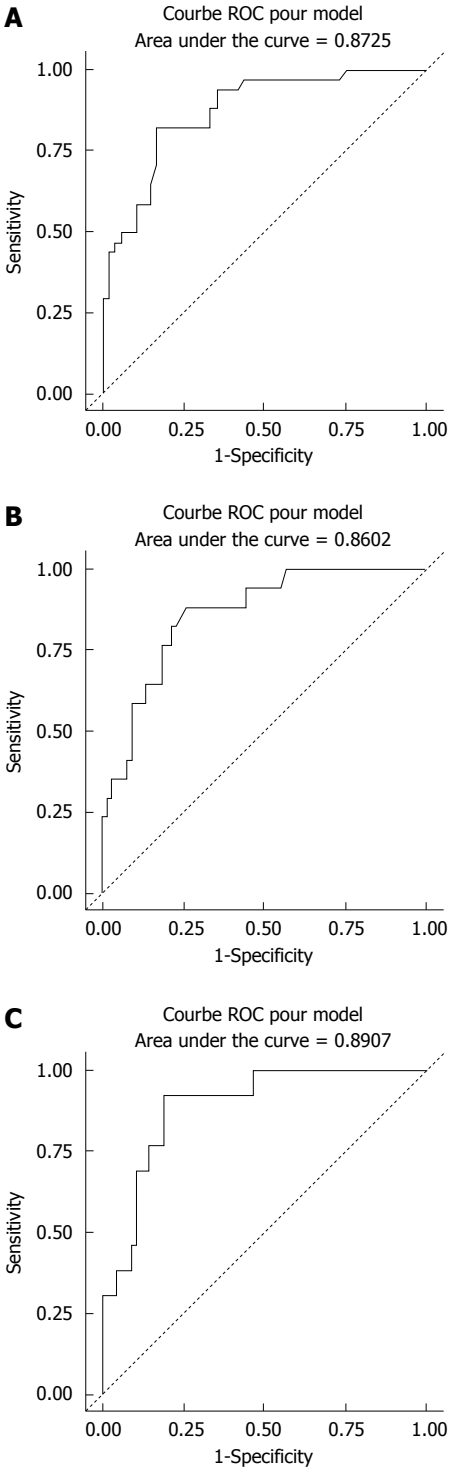

Figure 4 Receiver operating characteristic curves of acoustic radiation force impulse predicting liver fibrosis in patients with alcoholic liver disease. A: ROC curve for F ⩾ 2; B: ROC curve for F ⩾ 3; C: ROC curve for F = 4.

alternative for patients with contraindications to biopsy or TE. The ARFI measurement area size of 1 cm (compared to 4 cm for TE) can easily be offset by the possibility of several measurements in different parts of the liver.

Recently, studies have also started to evaluate ARFI on hepatitis B, hepatitis C and NASH[20-23]. ARFI has good intra-operator and inter-operator

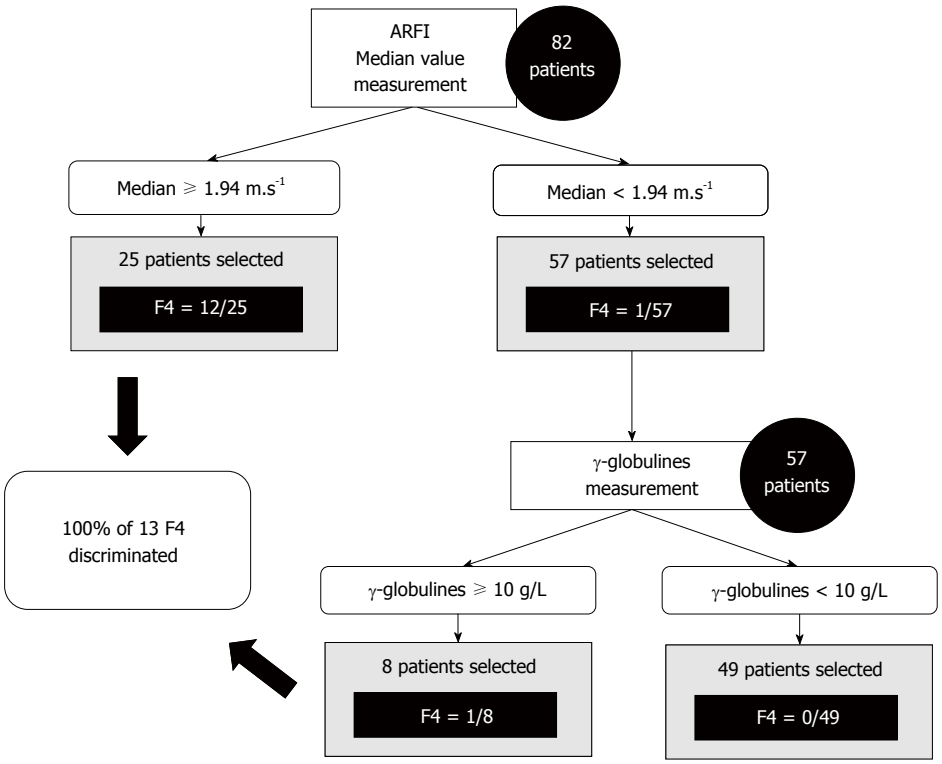

Figure 5 Decision tree for acoustic radiation force impulse predicting as a screening test for F = 4.

reproducibility, as described in the Bota *et al*[24] study, with an intraclass correlation coefficient (ICC) of 0.90 and 0.81 for intra- and inter-operator reproducibility respectively. The cut-off values reported in the literature are different, however. In hepatitis B, hepatitis C and NASH, cut-off values in m/s for F = 4 were respectively 1.84 for Dong *et al*[18] 1.55 for Sporea *et al*[21] and 1.9 for Yoneda *et al*[25]. These differences suggest that ARFI values differ depending on the disease, as shown in the meta-analysis by Nierhoff *et al*[23]. There is therefore a need to define cut-off values for each diffuse liver disease. To our knowledge, there is only one other study, by Zhang *et al*[26] evaluating the performance of ARFI imaging for the assessment of liver fibrosis in patients with ALD in comparison to biopsy, with an AUROC value of 0.89 for F = 4. However, the study populations are very different. In their international multi-centre study, Sporea *et al*[21] showed that the cut-off values predictive of fibrosis stages differ between European and Asian populations. This could explain why the cut-off values are respectively 1.27 and 1.65 for F ⩾ 2 and F = 4 in the Chinese study by Zhang and 1.63 and 1.94 in our study, using the same ultrasound device.

Our study showed good sensitivity and specificity, as described previously. But the excellent negative predictive value of ARFI (98.2% for F = 4) can open the possibility of using ultrasound elastography as a screening test rather than a diagnostic test. A decision tree of clinical value is proposed in Figure 5. Other larger studies are clearly needed to confirm these results.

Our study included patients undergoing alcoholic detoxification. Bardou-Jacquet *et al*[27] recently suggested that the alcohol consumption greatly influences TE and by extension liver stiffness, but could be a useful tool in the follow-up of patient as an indicator of alcohol consumption beyond the sole fibrosis evaluation. Fibrosis evaluation made on patients undergoing detoxification is the most common clinical situation. So, ARFI values may also be influenced by alcohol consumption and alcohol cessation. This may explain some mismatches between ARFI and biopsies.

In our study, ARFI was performed according to guidelines. In certain debatable conditions, the neutral condition was chosen. For example, according to the literature and the device provider's instructions, 10 measurements were taken and the median value was calculated, as for TE, with the patient gently holding their breath. Ten measurements were taken for each patient in our study, and the median was calculated for each one. Karlas *et al*[28] reported that deep inhalation on measurements could increase values by an average of 13%, while Horster *et al*[29] and Goertz *et al*[8] reported no difference. In our study, the patients were therefore asked to stop normal breathing for a moment. As previous studies reported that ARFI results could be influenced by food intake, we decided to perform ARFI in a fasted state[30]. An interlobar difference was found in the literature[31]. Our measurements were therefore taken in the right liver lobe in the intercostal space. This location was chosen for several reasons. First, operator pressure on the liver may produce false positives due to direct

probe compression, which occurs when measurements are taken in the left liver lobe. The measurements were taken in the intercostal spaces so that the ribs limit this compressive effect. Moreover, the operator exerted minimal pressure. Heartbeat artefacts could falsify the measurements when performed in the left lobe. Second, the aim was to use the same location as the biopsy. ARFI imaging was performed prior to liver biopsy to prevent the interaction of artefacts (such as from haematoma).

However, some findings are discordant with the results of the biopsy, which is considered the gold standard. One reason may be that ARFI produces mean values for a large area in the right liver lobe whereas liver biopsy involves taking a sample. The specimen obtained represents only 1/50000 of the total liver volume and it is well known that fibrosis has an uneven distribution within the liver[32]. In order to be comparable and reliable, multiple biopsies from different locations in the right liver lobe should be taken to gain an accurate comparison with the ARFI values obtained in different locations in the right liver lobe in the same intercostal space[33,34]. This requirement is ethically disputable. Another solution would be to compare ARFI values and hepatic explant findings.

In the literature, many factors have been reported to influence ARFI values, including sex, BMI, age, ethnicity, fasted state, depth of ROI, inflammation grade, obstructive cholestasis and certain other biological markers (alanine transaminase, platelets, prothrombin time, albumin, hyaluronic acid, cholesterol, γ-globulin)[18,21,28,30,35-41]. In our study, in the multivariate analysis, statistically significant correlations were only found for γ-globulin and age. This suggests that liver stiffness and hence fibrosis stage should be interpreted in view of the biological and clinical findings.

Millonig *et al*[42] suggested that liver stiffness is a direct function of central venous pressure and Goertz *et al*[8] reported that heart dysfunction may impair ARFI accuracy. None of the patients analysed in our study had heart failure.

In the literature, the median of the values is reported as being more accurate than the mean, and is used by convention. In our study we also calculated the mean of the 10 values for each patient. The correlation between the mean and median was almost perfect with a correlation factor of 0.98. This suggests that the mean of ten values could have been used instead of the median on our cohort of patients. Larger studies are needed to confirm this observation.

Another disputable point is the number of 10 values chosen for the median calculation. In many articles, the recommended number is 10. However, even if ARFI is a fast technique, obtaining ten values takes time. Therefore, in our study we analysed medians calculated from 2 to 9 values (the first values) and compared them to the median of 10 values. It would appear that a number of 6 values is sufficient to determine an accurate median with an excellent correlation coefficient of 0.98.

In addition to the benefits of ARFI as a non-invasive technique, our study has numerous strengths. This was a prospective study of a homogeneous population of alcoholic liver disease patients, with a delay no more than 10 d between the procedures and the beginning of alcohol withdrawal. The clinician, operator and pathologist were blinded to the results. Fibrosis was assessed by biopsy. The mean biopsy size was 30.7 ± 10.5 mm with the majority larger than 25 mm. Factors reported to influence ARFI results in the literature were taken into consideration and generally included in the multivariate analysis. Guidelines on the ARFI technique were summarised and applied to the measurements for each patient. To our knowledge, only one other study evaluating the performance of ARFI in predicting liver fibrosis in ALD has been published, but concerned a different ethnic population.

There were also limitations to our study. One is sample size. Larger studies or meta-analyses are needed to confirm the ARFI threshold in ALD. The comparison with TE was not done and would also be useful. The literature suggests that liver stiffness is influenced by inflammation[43,44]. Inflammation was only assessed and confirmed by transaminase levels in our study and not by histology. Correlation with steatosis grade was not assessed, but published data suggest that moderate/severe steatosis is not a significant error factor for ARFI elastography[14,18].

ARFI is an accurate, non-invasive and easy method for assessing liver fibrosis in patients with ALD. This imaging technique can be easily incorporated into routine patient care. Cut-off values are suggested and require further confirmation in larger studies. A comparison with TE and supersonic shear-wave elastography (Aixplorer Supersonic®) would be interesting for a complete live liver assessment.

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COMMENTS

Background

Acoustic radiation force impulse (ARFI) elastography has the particular advantage of combining conventional ultrasound and liver stiffness measurement. ARFI imaging is an accurate, non-invasive and easy method for assessing liver fibrosis in patients with hepatitis B and C. The aim of this study was to evaluate the performance of ARFI in determining fibrosis stage in patients with alcoholic liver disease (ALD).

Research frontiers

Liver biopsy is the gold standard for assessment of liver fibrosis. However, biopsy is an invasive procedure. This study suggests that ARFI imaging, a non-invasive method, could be used for the assessment of liver fibrosis in ALD.

Innovations and breakthroughs

The study showed the algorithm between ARFI and biochemical parameters for the prediction of presence of cirrhosis. Sensibility and specificity of ARFI were good. The investigation was carried out within European population of patients with ALD undergoing alcoholic detoxification.

Applications

This study is helpful for further research in Acoustic Radiation Force Impulse imaging among patients undergoing alcoholic detoxification.

Peer-review

The study showed the interesting algorithm between ARFI and biochemical parameters for the prediction of presence of cirrhosis. Interestingly, the investigation was carried out within European population of patients with ALD undergoing alcoholic detoxification. The paper makes original contribution and it is clinically exhaustive. The manuscript is well written, seems accurate and well organized. The authors clearly presented any doubts concerning the investigation conducted by them.

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ORIGINAL ARTICLE

Prospective Study

Longitudinal molecular characterization of endoscopic specimens from colorectal lesions

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Abstract

AIM: To compare molecular profiles of proximal colon, distal colon and rectum in large adenomas, early and late carcinomas. To assess feasibility of testing directed at molecular markers from this study in routine clinical practice.

METHODS: A prospective 3-year study has resulted in the acquisition of samples from 159 large adenomas and 138 carcinomas along with associated clinical parameters including localization, grade and histological type for adenomas and localization and stage for carcinomas. A complex molecular phenotyping has been performed using multiplex ligation-dependent probe amplification technique for the evaluation of CpG-island

methylator phenotype (CIMP), PCR fragment analysis for detection of microsatellite instability and denaturing capillary electrophoresis for sensitive detection of somatic mutations in *KRAS*, *BRAF*, *TP53* and *APC* genes.

RESULTS: Molecular types according to previously introduced Jass classification have been evaluated for large adenomas and early and late carcinomas. An increase in CIMP+ type, eventually accompanied with *KRAS* mutations, was notable between large adenomas and early carcinomas. As expected, the longitudinal observations revealed a correlation of the CIMP+/*BRAF*+ type with proximal location.

CONCLUSION: Prospective molecular classification of tissue specimens is feasible in routine endoscopy practice. Increased frequency of some molecular types corresponds to the developmental stages of colorectal tumors. As expected, a clear distinction is notable for tumors located in proximal colon supposedly arising from the serrated (methylation) pathway.

Key words: Colorectal cancer; CpG-island methylator phenotype; DNA; Microsatellite instability; *BRAF*

Core tip: The results indicate that molecular subtyping from endoscopic biopsies is feasible in routine gastroenterology practice to evaluate a patient's prognosis. Subtyping based on Jass classification can be used to evaluate molecular mechanisms of adenoma-carcinoma transition.

Minarikova P, Benesova L, Halkova T, Belsanova B, Suchanek S, Cyrany J, Tuckova I, Bures J, Zavoral M, Minarik M. Longitudinal molecular characterization of endoscopic specimens from colorectal lesions. *World J Gastroenterol* 2016; 22(20): 4936-4945 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i20/4936.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i20.4936

INTRODUCTION

The variability in clinical manifestation of colorectal cancer as well as considerable differences in outcome between some colorectal cancer patients has prompted wide-ranging research into the molecular basis of the disease[1]. The main effort has been directed at mechanisms underlying initiation and progression of colorectal neoplasia from normal colonic mucosa as well as factors defining therapy response and the overall patient's survival[2-5].

There is historic evidence suggesting that more than two-thirds of colorectal cancers begin as colorectal adenomas[6]. The size of adenoma is considered a fundamental risk factor and is directly associated with histological characteristics such as the amount of villosity and dysplasia. Aberrant activation of (proto)oncogenes in key signaling pathways has long been a subject of study in colorectal cancer research. Among others, mutations in two major (proto)oncogenes, *KRAS* and *BRAF*, are frequently found in both carcinomas as well as in adenomas[7]. In 1990, *KRAS* mutations were contributed to the shorter overall survival of colorectal cancer patients[8]. The prognostic value was later restricted only to specific *KRAS* mutation types (Exon 1, codon 12, but not codon 13 mutations)[9]. Later it was discovered that mutations in *KRAS* as well as *NRAS* (both members of a common subgroup, RAS-family) are the major causes of therapy resistance in colorectal tumors treated by monoclonal antiEGFR inhibitors[10,11]. Accordingly, the current NCCA guidelines include recommendations for predictive RAS-testing as a standard of care for colorectal carcinomas[12].

Since 1990, three distinct molecular pathways underlying the malignant transformation of advanced adenomatous polyps into cancerous lesions have been studied[13]. The different pathways are based on independent genomic events leading to the loss of key cellular regulatory mechanisms causing proliferation, invasion and metastasis. The resulting molecular subtypes are denoted by either chromosomal instability (CIN), microsatellite instability (MSI) or CpG-island methylator phenotype (CIMP)[14,15]. The subtypes are typically characterized by disruptions on the DNA level including mutations and allelic losses of major tumor suppressors in CIN[16], mutations of mismatch DNA repair genes in MSI[17] (also referred to as the replication of positive phenotype, RER+) and aberrant methylation of promoter regions of tumor suppressors in CIMP[18]. Over the past decade, clinical associations of these subtypes have been intensively studied. The majority of colorectal carcinomas bear signs of the CIN subtype, most notably somatic mutations of *APC* and *TP53* tumor suppressors and associated losses of alleles at 5q and 17p chromosomal locations [observed as a loss of heterozygosity (LOH)][19]. The CIN type is closely following the fundamental genetic model of colorectal tumorigenesis[20]. While the individual mutations and allelic losses of *APC* and *TP53* tumor suppressors bear no direct prognostic value[21], the "CIN high" phenotype derived from a combination of several markers (mutations and LOH) indicates poor survival compared to the "CIN low" or MSI phenotypes[22].

The CIMP phenotype is on the molecular level notably distinct from the CIN and may also be complemented by MSI[23,24] as a result of *MLH1* promoter methylation[25]. There is sufficient evidence that evaluation of CIMP together with *BRAF* mutation and combined with a presence or absence of MSI gives a strong indication of a patient's survival prognosis. Tumors bearing the CIMP+/*BRAF*+ phenotype exhibit

Table 1 Patient characteristics

Adenomas	94
Gender	
Women	39
Aged	34-98 (median 67.7)
Men	55
Aged	40-89 (median 68.0)
Localization	
Proximal colon	37
Distal colon	42
Rectum	15
Histology	
Tubular	47
Tubulovilous	39
Vilous	4
Serrated	4
Dysplasia	
Low-grade	78
High-grade	16
Carcinomas	127
Gender	
Women	44
Aged	34-98 (median 70.2)
Men	83
Aged	42-90 (median 68.5)
Localization	
Proximal colon	50
Distal colon	38
Rectum	39
Stage	
Early (Ⅰ and Ⅱ)	66
Advanced (Ⅲ and Ⅳ)	61

shorter disease-free survival[26]. Typically arising from serrated lesions and more frequent in the proximal colon (caecum and ascendens) they are the result of a specific molecular process and exhibit a distinct biological behavior[27]. In turn, a concurrent presence of MSI dramatically improves the prognosis of patients with CIMP+/*BRAF*+ tumors[28] as the MSI unstable tumors are less likely to spread to lymph nodes and to develop distant metastases[29]. Aside from the prognostic importance, there is also an ongoing discussion on the importance of CIMP/MSI/*BRAF* phenotyping for prediction of response to chemotherapy treatment[30].

In early 2015, two retrospective studies published a relationship between specific molecular subtypes and the survival of colorectal cancer patients on large patient cohorts[31,32]. Utilizing the knowledge of the above described molecular pathways, the specific molecular types were evaluated based on MSI and CIMP phenotyping in combination with the mutation status of *KRAS* and *BRAF*, as previously suggested by Jass[33]. A significant difference in survival for the different molecular types was indeed confirmed by both studies aimed at patients in stages Ⅲ and Ⅳ, respectively. The five molecular subtypes, now universally referred to as Type Ⅰ-Ⅴ, and a group consisting of the rest, marked as Others, were also characterized by their most likely longitudinal localization and the prevailing gender and age of the patients. Based on the studies mentioned above[31-33], Type 1 is characterized by CIMP+, *BRAF*+, MSI, proximal localization and good prognosis; Type 2 by CIMP+, *BRAF*+, microsatellite stability (MSS) or MSI-Low (MSI-L), proximal localization and poor prognosis; Type 3 by CIMP-, *KRAS*+, MSS or MSI-L, proximal localization and poor prognosis; Type 4 by CIMP-, *KRAS*- and *BRAF*-, MSS or MSI-L, distal localization and median prognosis; Type 5 by CIMP-, *KRAS*- and *BRAF*-, MSI, proximal localization and good prognosis.

While the original Jass characterization gave a unique complex view on the alternative pathways of molecular carcinogenesis, it has, most importantly, now been verified to represent a viable tool in clinical management of the disease. It is, therefore, eminent to adapt appropriate procedures for methodology as well as logistics of testing procedures in current clinical practice. While most studies traditionally rely on molecular testing directed at FFPE sections from resected tissue, endoscopic biopsies as well as endoscopically removed malignant polyps are also more recently being routinely used[34].

Longitudinal clinicopathological heterogeneity of colorectal cancer has been reported as early as 2002[35]. Biological diversity stemming from embryonic origins may be responsible for different mechanisms of tumorigenesis in proximal and distal colon and rectum resulting in different manifestation, response to therapy and the overall prognosis[36]. In this work, we present data from molecular phenotyping and mutation analysis of tissue samples acquired during colonoscopy. We present molecular profiling of colorectal carcinomas as well as of their precursor lesions, large adenomatous polyps. We evaluate molecular profiles at proximal, distal and rectal tumor localizations and assess overall feasibility and clinical utility of such molecular classification in routine endoscopy practice.

MATERIALS AND METHODS

Study population

The prospective study design was reviewed and certified by the Scientific and Ethics boards of the Military University Hospital. All patients admitted into the study have signed an informed consent. Patients were treated at the endoscopy unit and consecutive samples were collected during a 2-year prospective study. Tissue samples were obtained either as endoscopic biopsies or by endoscopic polypectomy (EPE) or endoscopic mucosal resection (EMR). The inclusion criteria was based solely on primary morphology evaluations by the endoscopist. The large adenomas (AA) were assigned as being any size greater than 1 cm[6]. Stage Ⅰ and Ⅱ carcinomas were jointly assigned as early carcinomas (EC) and Stage Ⅲ and Ⅳ were assigned as late

Table 2 Overview of the molecular testing results *n* (%)

Marker	Localization	Advanced adenoma[1]	Early carcinoma[2]	Late carcinoma[3]
MSI	Proximal	0 (0)	5 (20)	7 (24.14)
	Distal	0 (0)	0 (0)	0 (0)
	Rectum	0 (0)	0 (0)	0 (0)
CIMP	Proximal	13 (30.95)	15 (65.22)	16 (59.25)
	Distal	7 (16.28)	10 (45.45)	6 (35.29)
	Rectum	2 (13.33)	11 (50.22)	8 (47.06)
BRAF	Proximal	7 (10.94)	4 (17.39)	7 (24.14)
	Distal	1 (1.49)	0 (0.00)	0 (0.00)
	Rectum	4 (17.39)	1 (4.20)	2 (11.11)
KRAS	Proximal	25 (35.71)	11 (44.00)	15 (51.72)
	Distal	28 (43.08)	11 (44.00)	4 (23.53)
	Rectum	12 (50.00)	10 (41.67)	7 (38.88)
APC	Proximal	24 (35.82)	5 (20.83)	5 (17.24)
	Distal	22 (34.38)	8 (34.78)	6 (37.5)
	Rectum	9 (37.50)	7 (29.17)	8 (44.44)
TP53	Proximal	5 (7.46)	8 (33.33)	8 (27.59)
	Distal	2 (3.13)	8 (34.78)	8 (50.00)
	Rectum	1 (4.17)	11 (45.83)	10 (55.55)

[1]> 1 cm; [2]Stage Ⅰ or Ⅱ; [3]Stage Ⅲ or Ⅳ.

carcinomas (LC). The description of patients from this study is listed in Table 1.

Tumor characteristics

In order to follow a prospective strategy of all evaluations, we have decided to use adenomatous polyp size beyond 10 mm as the only inclusion criteria that allows immediate decision about molecular testing during the endoscopy procedure. DNA from fresh biopsies or FFPE sections was extracted following a standard histopathology evaluation to ensure adequacy (viability, quantity, tumor cell fraction) for the testing. On FFPE sections, tumor-positive areas were clearly marked by a pathologist prior to microdissection. DNA was extracted from fresh and FFPE specimens using a standard spin-column procedure using a commercial kit (JETquick Tissue DNA spin, GENOMED G.m.b.H, Loehne, DE).

Microsatellite instability testing

Microsatellite instability was evaluated using MSI Analysis System, Version 1.2 (Promega corporation, Madison, WI, United States). The multiplex PCR kit produces fluorescently labelled amplicons of five nearly monomorphic mononucleotide markers (BAT-25, BAT-26, NR-21, NR-24 and MONO-27) and two additional polymorphic markers (Penta C and Penta D) for specimen identification[37]. PCR amplicons were resolved on a 16-capillary sequencer (ABI PRISM 3100, Applied Biosystems, Foster City, CA, United States) according to the manufacturers protocol. The data was evaluated by GeneMarker software (Softgenetics, State College, PA). Only samples exhibiting unstable alleles at 2 or more markers were assigned as MSI, otherwise the assignment was MSI-L (1 marker instable) or MSS (no unstable markers detected).

CpG island methylator phenotype testing

The CIMP phenotype evaluation was based on multiplex ligation-dependent probe amplification technique (MLPA) utilizing a non-bisulfite conversion approach. A commercial MLPA kit was used (SALSA MLPA ME042 CIMP, MRC Holland, NL) and the MLPA data was evaluated by GeneMarker software using an appropriate MLPA CIMP panel (available for download from the Softgenetics website). The investigated genes were as suggested by Ogino[38]. A CIMP-high phenotype was assigned to a sample showing any of the MLPA probes methylated for at least 6 out of 8 evaluated genes (*RUNX3*, *CACNA1G*, *IGF2*, *MLH1*, *NEUROG1*, *CRABP1*, *SOCS1* and *CDKN2A*)[39].

KRAS, BRAF, APC and TP53 mutation testing

Somatic mutation testing in *KRAS, BRAF, APC* and *TP53* genes was performed by denaturing capillary electrophoresis (DCE) using a previously described protocol[40-43]. The technique is based on a principle of differential denaturation of wildtype and mutant alleles, similar to the high-resolution melting technique[44]. In brief, the target sequences harboring the mutation sites were amplified using GC-clamping at one of the primers and a fluorescence label at the other primer. The PCR amplification program was concluded by a heteroduplex formation step in which the product mixture was heated for 8 min at 95 ℃, then kept at 65 ℃ for 30 min and finally cooled at 0.1 ℃/s down to 15 ℃. Each amplicon was then subjected to capillary electrophoresis separation at optimized separating temperature leading to the resolution of homo- and hetero- duplex forms in case of a mutation presence. In order to speed up the screening process, amplicons with similar separating temperatures were analyzed in different capillaries during the same run. The target amplicons included exons 2, 3 and 4 of *KRAS* gene, the V600E mutation (exon 15) of *BRAF* gene[41], codon span 1250-1550 (mutation cluster region) of *APC* gene[42,45] and exons 5 to 8 of *TP53* gene[43]. According to the Catalog of somatic mutations in cancer (COSMIC) this testing panel should detect more than 88% of somatic mutations in the studied genes[46].

RESULTS

Over the 2-year duration of the project, a total of 6080 colonoscopies were performed yielding 297 tissue specimens. The set included 159 large AA, 74 EC and 64 LC (see Methods for details of the AA/EC/LC assignment).

The success rates for DNA extractions were 96.3% (104/108) for fresh tissue and 93.7% (177/189) for FFPE sections. The amounts of extracted DNA were typically between 500-1000 μL volumes of 5-10 ng/μL. A complete set of results consisting of MSI, CIMP, *BRAF*, *KRAS*, *APC* and *TP53* data was obtained for 246 out of 281 extracted DNA samples (87.6%). The

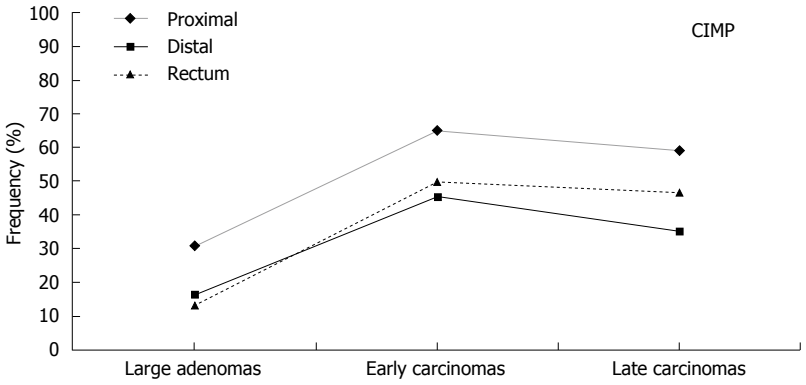

Figure 1 Longitudinal frequency of CpG-island methylator phenotype phenotype in different tumor types. CIMP: CpG-island methylator phenotype.

incomplete molecular profiles were largely due to failed CIMP examination in FFPE mainly as a results of low amounts or low quality of DNA. Results for individual markers obtained for each tumor subtype at proximal, distal and rectal localizations are listed in Table 2.

CIMP, BRAF and MSI

The distribution of CIMP+ phenotypes for the three evaluated tumor types along the proximal and distal colon and rectum is shown in Figure 1. In all three types the CIMP+ frequency in proximal colon is 15% higher than in distal colon or rectum. In all three sections there is a 2-3 fold jump in frequency between large adenomas and early carcinomas while only a relatively small change (< 10%) between early and late carcinomas.

The *BRAF* mutations were found in 12 of 154 large adenomas (7.8%), 5 of 74 (6.8%) early carcinomas and in 9 of 64 (14.1%) advanced carcinomas. A CIMP+/*BRAF*+ combination was mostly found in proximal colon with frequency gradually increasing with the tumor progression from 5.3% (2/38) in large adenomas to 13% (3/23) and 26% (7/27) in early and late carcinomas, respectively.

In agreement with previous reports MSI has only been found in early and late cancers, but not in adenomatous tissue[47]. In carcinomas, MSI was detected only in the proximal localization at 16.0% in early cancers (4/25) and 24.1% in late cancers (7/29). MSI was accompanied by CIMP+ phenotype in 81.2% (9/11) and 88.9% (8/9) of CIMP+ carcinoma had *MLH1* promoter methylation.

APC, KRAS and TP53

Mutations in *APC*, *KRAS* and *TP53* were observed in all tumor groups across proximal and distal colon as well as in the rectum. Similarly to a recently published study[25], we have found a higher frequency of *APC* and *KRAS* mutations in CIMP+ carcinomas with a presence of *MLH1* methylation when compared to CIMP+ without *MLH1* methylation. The difference was 20%; 2/10 *vs* 33.3%; 9/27 for *APC* (P = 0.74) and 21.4%; 3/14 *vs* 59.3%; 16/27 for *KRAS* (P = 0.031). Regardless of the tumor localization, *TP53* mutation rates showed a significant increase from large adenomas (5.1%; 8/155) to early and late carcinomas (36.5%; 27/74 for early and 41.3%; 26/63 for late, $P < 0.001$, $\chi^2 = 49.928$). Also in an agreement with previous findings[48] *TP53* mutations were detected more frequently in the group of CIMP- carcinomas compared to the CIMP+ carcinomas (39.6%; 38/96 *vs* 27.1%; 13/48), but the result was not statistically significant.

DISCUSSION

Principal contributions of various pre-analytical factors to the success of molecular genetic testing from FFPE sections have long been studied[49]. Among others, the principal importance of the quality of the formalin solution (buffered to neutral pH) and the duration of fixation has been recognized[50]. The negative effects of fixation are intensified for small volume samples, typically acquired by endoscopic biopsies. At the same time, upon extraction, the small biopsy specimens often yield low amounts of DNA limiting the extent of the molecular testing. For complex molecular profiling, such as the subtyping performed in this study, a prioritization of the individual tests, as already practiced in molecular testing of other cancer types[51], is clearly a necessity for future routine use.

Most cases of inconclusive results in this study were, indeed, due to the low DNA quality or amount. A dedicated mutation technology typically based on single-plex PCR usually requires only minute amounts of DNA. The MSI detection approach utilizing a multiplex PCR followed by capillary electrophoresis is also low to medium in the demand of DNA. On the other hand, CIMP evaluation by MLPA requires by far the highest amounts of input DNA. With a very limited availability of other reliable CIMP-detection techniques, this is clearly the limiting factor.

Assignment of Jass molecular subtypes

According to the original work of Jass[33] and the recent publications by Phipps *et al*[32] and Sinicrope

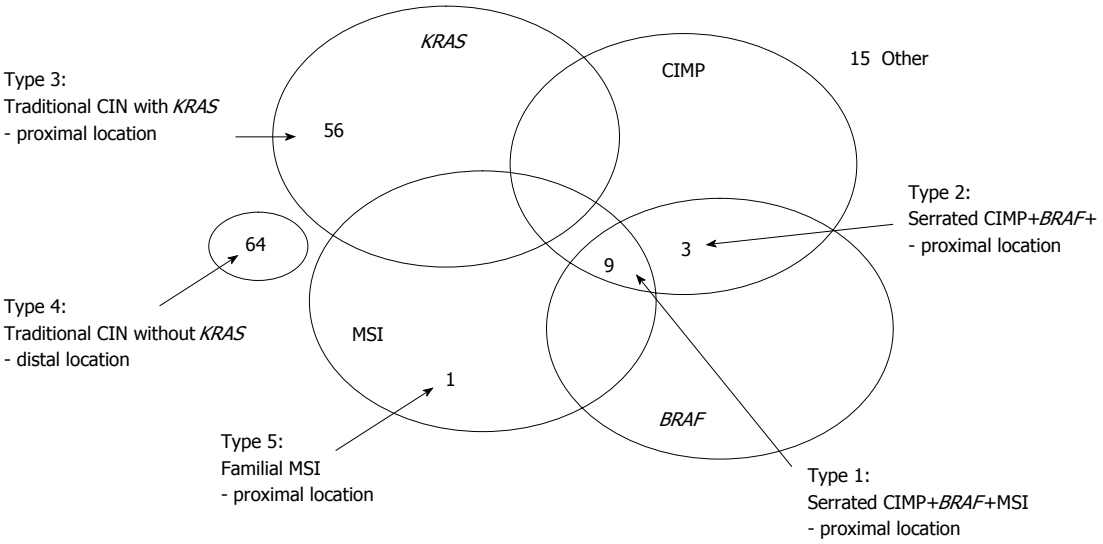

Figure 2 Molecular classification of colorectal carcinomas (all stages) using classification according to Jass and others[30-32]. MSI: Microsatellite instability; CIN: Chromosomal instability; CIMP: CpG-island methylator phenotype.

et al[31], we have applied their principles to our data to assign the molecular subtypes. The classification is based on a combined evaluation of CIMP/MSI/*BRAF*/*KRAS* testing. The resulting spectrum of molecular subtypes for carcinomas in our study is presented in Figure 2. Even with the smaller size of our prospective group, the relative distribution among the 6 different groups (Types 1-5 and Others) corresponds to the data presented in those large retrospective cohorts. The Type 4 and Type 3, both characteristic of the CIN pathway, were the most frequent at 43.2% and 37.8%, respectively, followed by Types 1 and 2, resulting from the CIMP-serrated pathway, at 6.0% and 2.0%, respectively.

The probability of developing future advanced adenomas or cancers increases with the size of adenoma and can range from 1.5% to 7.7% for sizes below 5 mm, 3% to 15.9% for sizes between 5 and 20 mm and 7% to 19.3% for adenomas over 20 mm in size[6]. We have evaluated the Jass-types separately for the groups of large adenomas, early carcinomas and late carcinomas to visualize the degree of molecular irregularities along the tumor progression route. The evaluation workflows for all groups are shown in Figure 3.

A notable change in the distribution patterns of the molecular types can be observed between large adenomas and early carcinomas. The main difference appears to be a result of an increase in CIMP+/*BRAF*- phenotypes from large adenomas (13.8%, 13/94) to early carcinomas (50.0%, 31/62). When explored further, an additional increase in a *KRAS* positive subgroup can be noticed. Accordingly, the rate of CIMP+/*BRAF*-/*KRAS*+ increases from 10.6% (10/94) in large adenomas to 30.6% (19/62) in early carcinomas. At the same time, this increase is complemented by the decrease of CIMP-/*BRAF*-/*KRAS*+ from 49.0% (38/94) in large adenomas to 16.1% (10/62) in early carcinomas, but also partially by the decrease in CIMP-/*BRAF*-/*KRAS*- from 39.3% (37/94) in large adenomas to 30.6% (19/66) in early carcinomas. In other words, methylation, partially accompanied by *KRAS* mutation, takes place during malignant transformation of at least some colorectal tumors during the transition from large adenomas to early carcinomas.

In addition to the Jass types an interesting molecular subgroup has recently been identified including carcinomas with CIMP+ phenotype with unmethylated *MLH1* harboring *KRAS* mutations[25]. We have identified high frequency of *KRAS* mutations in the CIMP+/unmethylated *MLH1*- group within early carcinomas (10/17; 58.8%) as well as late carcinomas (6/10; 60%). According to the previous reports such cancers arise mainly from *KRAS*-mutated traditional serrated adenomas and exhibit poor prognosis. This is in contrast to the CIMP- carcinomas.

Characterization of molecular types according to location

Combined with the information on mutator pathways a full longitudinal image of the colorectal cancer landscape can be elucidated[52]. Data from our study have confirmed the predominant manifestation of the CIMP-associated Type 1 and Type 3 in the proximal colon. At the same time, tumors bearing the CIN characteristics are evenly distributed throughout the colon and rectum. It is clear that further research will lead to more molecular tests to be performed routinely in the diagnosis and therapy of colorectal neoplasia. The molecular subtyping of adenomas and carcinomas using the Jass classification may lead to the discovery of molecular markers specific for the malignant conversion of colonic tissue from precursor lesions to malignant tumors. Such markers would be viable tools to complement endoscopic screening and the diagnosis of colorectal cancer patients.

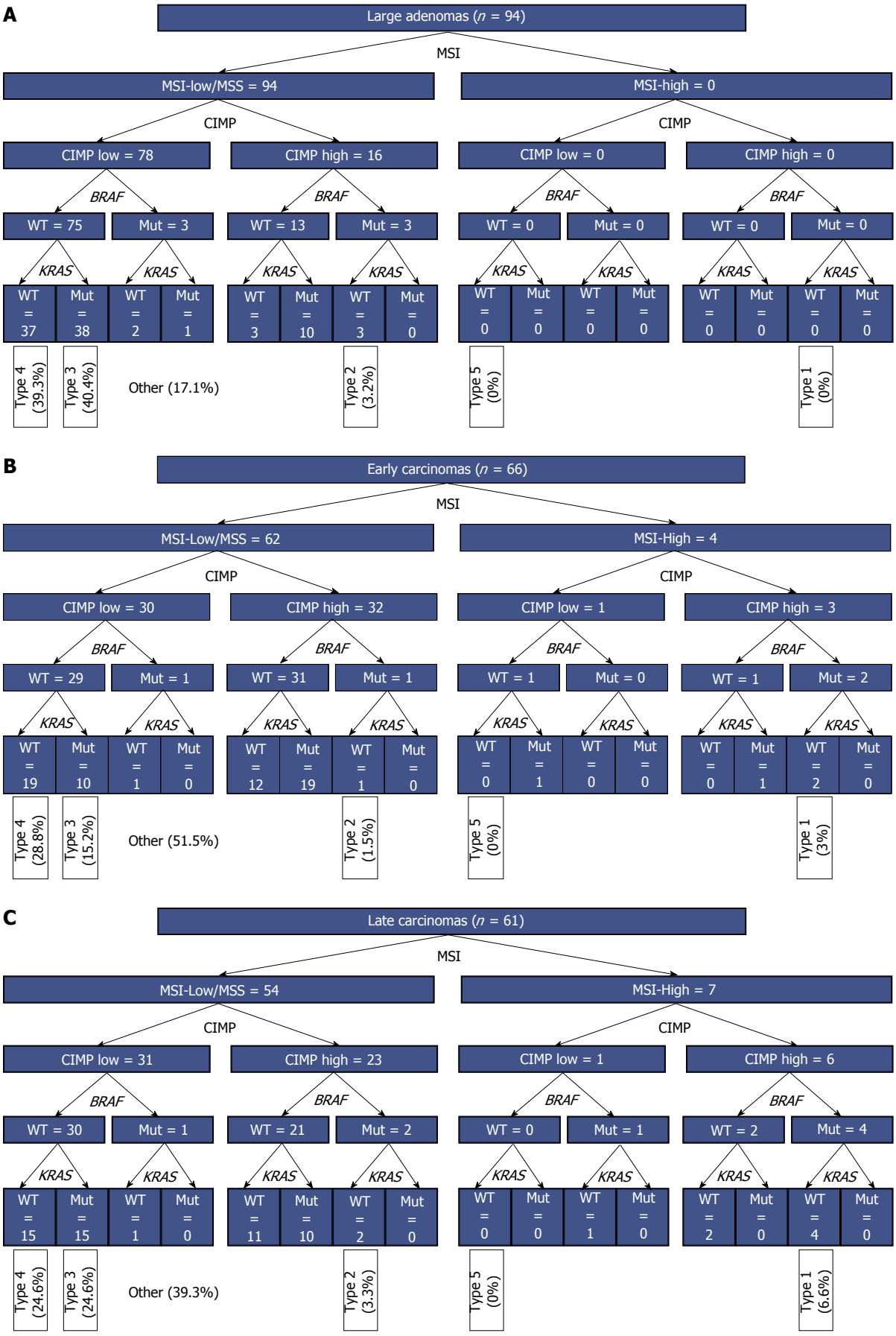

Figure 3 Evaluation workflows for assignment of Jass types in large adenomas (A), early carcinomas (B) and late carcinomas (C).

COMMENTS

Background

Recent advances in molecular profiling have resulted in definition of molecular types of colorectal cancer based on genetic and epigenetic aberrations. Resulting from separate developmental pathways the different types are associated with distinct prognostic features, which can be utilized in clinical practice.

Research frontiers

In a prospective study, endoscopic specimens from colorectal carcinomas as well as pre-malignant lesions were subjected to molecular profiling directed at evaluation of microsatellite instability (MSI) and CpG-island methylator phenotype (CIMP) status in combination with somatic mutations of *KRAS, BRAF, TP53* and *APC* genes.

Innovations and breakthroughs

The distribution of molecular types was evaluated for precursor lesions (large adenomas) and for early and late carcinomas with respect to their localization in proximal colon, distal colon and rectum.

Applications

The study demonstrates feasibility of molecular profiling in routine gastroenterology practice. The study results further suggest distinct molecular changes occurring during the malignant transition from large adenoma to early carcinoma, in particular DNA methylation affecting *KRAS*-mutated tumors.

Terminology

Somatic aberrations: Changes in DNA composition (base sequence or methylation) occurring within cells as a result of external factors and not the inheritance. CIMP: A molecular subtype characterized by methylation at certain positions within the DNA sequence. MSI: A molecular subtype characterized by unequal numbers of repetitions of short DNA sequences obtained for different cells within a tissue. The MSI occurs due to somatic aberrations in genes securing a proper function of the DNA repair system. Promoter methylation of *MLH1* gene is a frequent cause of MSI.

Peer-review

The authors studied molecular profiles of proximal and distal colon and rectum in colorectal adenomas and carcinomas that were obtained by routine endoscopic biopsy. They analyzed CIMP, MSI and mutations of *KRAS* and *BRAF*, and then classified into molecular subtypes in colorectal tumors. Most importantly, longitudinal molecular characterization was clearly shown in colorectal tumors based on CIMP/MSI/*BRAF*/*KRAS* classification. This approach to the molecular classification of colorectal cancer should accelerate understanding of causation, have an impact on clinical management, and facilitate the development of new ways to prevent and treat colorectal cancer.

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SYSTEMATIC REVIEWS

Colorectal cancer screening in countries of European Council outside of the EU-28

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Abstract

AIM: To provide an update on colorectal cancer (CRC) screening programmes in non-European Union (EU)-28 Council of Europe member states as of December 2015.

METHODS: The mission of the Council of Europe is to protect and promote human rights in its 47 member countries. Its 19 non-EU member states are Albania, Andorra, Armenia, Azerbaijan, Bosnia and Herzegovina, Republika Srpska, Georgia, Iceland, Liechtenstein, Republic of Moldova, Monaco, Montenegro, Norway, Russian Federation, San Marino, Serbia, Switzerland, FYR of Macedonia, Turkey, and Ukraine (EU-19). The main data source were GLOBOCAN, IARC, WHO, EUCAN, NORDCAN, ENCR, volume X of the CI5, the ministerial and Public Health Agency websites of the individual countries, PubMed, EMBASE, registries of some websites and the www.cochranelibrary.com, Scopus, www.clinicaltrials.gov, www.clinicaltrialsregister.eu, Research gate, Google and data extracted from screening programme results.

RESULTS: Our results show that epidemiological data quality varies broadly between EU-28 and EU-19 countries. In terms of incidence, only 30% of EU-19 countries rank high in data quality as opposed to 86% of EU-28 states. The same applies to mortality data, since 52% of EU-19 countries as against all EU-28 countries are found in the high ranks. Assessment of the method of collection of incidence data showed that only 32% of EU-19 countries are found in the top three quality classes as against 89% of EU-28 countries. For the mortality data, 63% of EU-19 countries are found in the highest ranks as opposed to all EU-28 member states. Interestingly, comparison of neighbouring countries offering regional screening shows, for instance, that incidence and mortality rates are respectively 38.9 and 13.0 in Norway and 29.2 and

10.9 in Sweden, whereas in Finland, where a national organised programme is available, they are respectively 23.5 and 9.3.

CONCLUSION: Cancer screening should be viewed as a key health care tool, also because investing in screening protects the weakest in the population, decreases the social burden of cancer, and reduces all types of health care costs, including those for radical surgery, long-term hospitalisation, and chemotherapy.

Key words: Colorectal cancer; Screening; EU-28; EU-19; European Union; Early detection; European Council

Core tip: In the WHO Europe Region, colorectal cancer (CRC) is the first tumour with 471000 new cases per year and a mortality rate of 28.2 per 100000 population. Large-scale studies have found a reduction in mortality due to the adoption of population-based screening programmes. A 2010 European Parliament resolution called for the adoption of prevention programmes. As a result, some member states have begun enacting programmes, others are organising strategies for CRC screening implementation, and others still are moving from pilot projects to national-scale programmes. The present systematic review provides an update on CRC screening programmes in non EU-28 European Council States.

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INTRODUCTION

Although cervical, breast and colorectal cancer are the only tumours for which screening has proven efficacy and cost-effectiveness, in several European countries screening implementation is fraught with difficulties. This is especially true of programmes regarding colorectal cancer (CRC)[1-3], a highly common malignancy. According to GLOBOCAN data[3], 1.36 million new cases affecting 17.2 per 100000 population (746000 men and 614000 women) are diagnosed in the world each year, and 693000 people (373000 men and 320000 women) die from CRC, accounting for a yearly mortality rate of 8.4 per 100000.In the World Health Organisation (WHO) Europe Region, CRC is the first tumour by incidence, with 471000 new cases each year and a mean mortality rate of 28.2 per 100000 population[4]. In the European Union (EU-28), its mean incidence rate is 31.3 per 100000 population, with 345000 new cases per year and an incidence per 100000 population of 39.5 for men 39.5 and 24.4 for women. The mean CRC incidence rates for men and women in the WHO Europe Region are 35.6 and 22.6 per 100000 population, respectively. In addition, with 228000 deaths per year and a mortality rate of 12.3 per 100000 population, CRC is the second cause of cancer death after lung cancer for men and women in the region[4]. The mean mortality rates per 100000 population in EU-28 countries and the WHO Europe Region are respectively 15.2 and 15.7 for men and 9.0 and 9.7 for women[4].

CRC incidence is quite variable in EU-28 countries, and is higher in central and northern member states than in eastern ones. However, the lower rates found in eastern Europe are higher than the world mean[3]. This has prompted the Council of Europe to recommend the priority activation of CRC screening programmes[5]. According to a 2008 European Commission report on the diffusion of CRC screening programmes in the EU, only 12 of the then 22 member states had population-based screening programmes; the others were recommended to provide to their citizens equal access to cancer prevention[6].

Crucially, more than 95% of CRC cases could benefit from surgical treatment if diagnosed early[7]. Several large-scale studies have found a considerable reduction in mortality due to the adoption of population-based screening programmes[8,9].

The first European guidelines on CRC screening and the quality of CRC diagnosis were issued in 2010[10]. A European Parliament resolution of 6 May 2010 asked the Commission to promote the adoption of prevention programmes by any means and to encourage member states to allocate further resources to primary prevention and early diagnosis through screening[11]. As a result, some member states have begun enacting programmes, others are organising strategies for CRC screening implementation[3], and others still are moving from pilot projects to national-scale programmes[12-16].

The aim of the present systematic review is to provide an update on CRC screening programmes in non EU-28 European Council member states as of December 2015.

MATERIALS AND METHODS

Council of Europe member countries

The Council of Europe is a supranational institution founded in 1949 by the Treaty of London. Its mission is to protect and promote human rights in member countries. There are 47 member countries and a number of states with observer status. All EU-28 States are members (Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, the Netherlands, and the United Kingdom). The other

Table 1 Number of cases and standardized colorectal cancer burden in women and men in EU-19, 2012 (Adapted from Ferlay *et al*[4])

Country	WOMAN						MAN					
	Incidence		5-yr prevalence		Mortality		Incidence		5-yr prevalence		Mortality	
	Cases	ASR (W)	Cases	%	Cases	ASR (W)	Cases	ASR (W)	Cases	%	Cases	ASR (W)
Albania	167	7.9	501	39.9	90	4.0	175	9.0	526	42.0	97	4.8
Andorra	NR											
Armenia	4.4	17.0	939	68.6	281	9.7	426	22.8	855	77.1	261	13.4
Azerbaijan	346	6.4	209	4.0	755	19.8	313	7.1	661	18.5	187	4.3
Bosnia and Herzegovina	489	13.3	1427	84.8	327	7.7	620	20.7	1811	119.1	422	12.7
Georgia	300	7.5	608	31.3	173	4.0	305	9.9	631	38.3	177	5.5
Iceland	79	28.3	238	183.6	21	5.8	78	28.9	232	177.5	27	9.3
Liechtenstein	NR											
Republic of Moldova	716	23.0	1736	111.0	409	12.6	799	36.0	1963	143.2	491	22.0
Monaco	NR											
Montenegro	107	21.1	314	118.7	67	12.0	157	36.2	465	187.3	95	20.7
Norway	1947	35.8	5665	279.6	779	12.1	1966	42.6	5839	289.8	727	14.3
Russian Fed	33183	21.8	78454	119.1	21791	12.7	26745	30.0	63572	116.4	18116	19.9
San Marino	NR											
Serbia	2143	23.3	6281	151.4	1213	11.5	3370	43.4	9919	248.8	1922	22.8
Switzerland	2167	23.6	6522	193.8	718	6.4	2707	36.3	8340	259.9	1668	12.8
FYR Macedonia'	366	20.5	1070	124.0	213	10.8	421	28.4	1256	147.3	239	15.5
Turkey	5041	13.1	10690	38.2	3030	7.8	6889	20.6	14982	54.7	4128	12.6
Ukraine	9780	19.9	23110	109.6	5704	10.8	6269	29.9	22120	127.8	5929	18.8
EU-28	151920	24.4	417252	189.0	69087	9.0	193426	39.5	535845	257.8	82959	15.2

EU-28: Countries members of European Union; NR: Not Reported; ASR (W): Per 100000.

19 countries (hereafter EU-19) are in the European area: Albania, Andorra, Armenia, Azerbaijan, Bosnia and Herzegovina, Republika Srpska, Georgia, Iceland, Liechtenstein, Republic of Moldova, Monaco, Montenegro, Norway, Russian Federation, San Marino, Serbia, Switzerland, FYR of Macedonia, Turkey, and Ukraine.

Sources of EU-19 epidemiological data: Search strategy

The main data source was the GLOBOCAN 2012 website of the International Agency for Research on Cancer (IARC), which provides access to several databases that enable assessing the impact of CRC in 184 countries or territories in the world[4].

Additional sources were the WHO, EUCAN and NORDCAN, the European Network of Cancer Registries (ENCR), volume X of the CI5, and the ministerial and Public Health Agency websites of the individual countries. The PubMed search used "Early Detection of Cancer" or "Colorectal Cancer screening" AND "state name" for each of the 19 countries. A MeSH search was conducted using the same criteria. The EMBASE did not provide further relevant results. The registries of some websites and the www.cochranelibrary.com, Scopus, www.clinicaltrials.gov, www.clinicaltrialsregister.eu, Research gate, and Google databases were also consulted. Other data were extracted from screening programme results.

Statistical analysis

Incidence and mortality data, their age-standardised rates per 100000 population (ASR-W), and 5-year prevalence estimates for 2012 are reported by gender in Table 1. The quality of incidence and mortality data of EU-19 and EU-28 based on Data Sources and Methods[17] is compared in Table 2. The information regarding screening programmes in EU-19 is shown in Table 3. Finally mean income, total population, the existence of any registries, the availability of early detection tests at the public primary health care level, and the ranking of CRC incidence and mortality in EU-19 countries are reported in Table 4. The distribution of screening programmes (organised, spontaneous, unknown) in EU-28 and EU-19 countries is shown in Figure 1.

RESULTS

The results of the present systematic review are listed by physical geographical area as well as disaggregated by state. The incidence and mortality data are reported as ASR-W per 100000 population.

Northern Europe

The only North European countries that are not also EU-28 members are Iceland and Norway. The United Kingdom and Northern Ireland, Ireland, Finland, Denmark, Estonia, and Latvia offer organised national screening programmes and Sweden an organised regional programme; only Lithuania adopts spontaneous screening (Figure 1).

Iceland: The incidence rate of CRC in Iceland is 28.9 and 28.3 in men and women, respectively, with

Table 2 Quality assessment of Epidemiological data source and methods according to Mathers *et al*[17]

Data source			
EU-19 Incidence A: Iceland Norway, Ukraine B: Serbia, Switzerland C: Turkey D: Bosnia Herzegovina, Russian Federation E: - F: - G: Albania, Armenia, Azerbaijan, FYR Macedonia, Georgia, Montenegro, Republic of Moldova	EU-19 Mortality 1: Iceland, Republic of Moldova 2: Azerbaijan, Norway, Russian Federation, Serbia, Switzerland 3: Albania, Armenia, FYR Macedonia 4: - 5: Bosnia Herzegovina 6: Georgia, Montenegro, Turkey	EU-28 Incidence A: Austria, Belgium, Bulgaria, Croatia, Czech Republic, Denmark, Estonia, Ireland, Latvia, Lithuania, Malta, Sweden, Slovenia,Slovakia, The Netherlands, United Kingdom, Finland, France (Martinique) B: France, Germany, Italy, Spain C: Portugal, Poland D: Luxembourg, France (La Reunion) E: Romania F: - G: Greece, Hungary, France (Guadalupe), France (Guiana)	EU-28 Mortality 1: Estonia, Hungary, Ireland, Latvia, Lithuania, Malta, Slovenia, Slovakia, Romania, United Kingdom, Finland 2: Austria, Belgium, Bulgaria, Croatia, Czech Republic, Denmark, France, Germany, Italy, Luxembourg, Spain, Sweden, The Netherlands, France (Guadalupe), (La Reunion), France (Martinique), France (Guiana) 3: Greece, Portugal, Poland 4: - 5: - 6: -
Methods			
EU-19 Incidence 1: Iceland, Norway 2: Bosnia Herzegovina, Ukraine 3: Turkey, Switzerland 4: Albania, FYR Macedonia, Republic of Moldova, Serbia 5: Armenia, Azerbaijan, Georgia 6: Turkey 7: - 8: - 9: Montenegro	EU-28 Mortality 1: Albania, FYR Macedonia, Iceland, Norway, Republic of Moldova, Serbia, Switzerland 2: Armenia, Azerbaijan, Bosnia Herzegovina, Georgia, Ukraine 3: - 4: - 5: Turkey 6: Montenegro	EU-19 Incidence 1: Austria, Bulgaria, Croatia, Czech Republic, Denmark, Estonia, Finland, Germany, Ireland, Latvia, Lithuania, Malta, Slovakia, Slovenia, Sweden The Netherlands, United Kingdom, France (La Reunion), France (Martinique) 2: Belgium, Cyprus 3: France (metropolitan) , Germany, Italy, Poland, Spain 4: Greece, Hungary, Luxembourg, Portugal 5: Romania, France (Guadalupe), 6: - 7: - 8: - 9: -	EU-28 Mortality 1: Austria. Bulgaria, Croatia, Czech Republic, Denmark, Estonia, Finland, France (metropolitan) Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Poland, Portugal, Slovakia, Slovenia, Spain, Sweden, The Netherlands, United Kingdom, France (Martinique) 2: Belgium, Cyprus, France (Guadalupe) 3: Greece, Portugal, Poland 4: - 5: France (La Reunion) 6: -

Comparison between EU-19 and EU-28 countries, as defined in the main text. -: No country classified in that category.

a mortality rate of 9.3 for men and 5.8 for women (Table 1).The national cancer registry, linked to the NORDCAN project, covers the whole population and provides high-quality data (Table 2). Iceland has no active organised CRC screening programme (Table 3). The decision to adopt one, made in 2008[18], was postponed due to the economic crisis. According to a recent congress communication[19], a programme offering screening with the iFOBT at 2-year intervals to 55 to 75 year olds is due to start soon (Table 3). Until then, only spontaneous screening with the iFOBT will be available at the level of public primary health care (Table 4). CRC is the third most common tumour in both genders in the country and the fourth and second cause of cancer death in Iceland (Table 4).

Norway: In this country the incidence of CRC is 42.6 among men and 35.8 among women, with a mortality rate - 12.1 in men and 14.3 in women (Table 1). High data quality is ensured by a national cancer registry linked to the NORDCAN that covers the whole population (Table 2). A pilot study offering the iFOBT at 2-year intervals was activated in 2012 in the Ostfold region[20]. In a randomised controlled study (NORCCAPP) conduced in the Oslo and Telemark areas in 1999-2001 the population was assigned to three groups that were tested with the iFOBT, received the iFOBT + sigmoidoscopy, or were just asked to report if they had had a diagnosis of CRC in the course of the study[21] (Table 3). CRC is the second most common tumour in both sexes and the second cause of cancer death for both sexes in Norway (Table 4).

Balkan countries

Several of these countries are EU-19 States: Albania, Republika Srpska, Bosnia and Herzegovina, Montenegro, and Serbia. Slovenia and Croatia are EU-28 Member states offering organised screening programmes (Figure 1).

Albania: Albania has a low CRC incidence rate, 9.0 among men and 7.9 among women, and an equally low mortality rate, respectively 4.8 and 4.0 (Table 1). Hospital-based disease registries provide non-excellent data quality (Table 2). Neither spontaneous nor organised screening is available[22]. The most recent data are for 2011. A 2015 paper[23] that first measured the frequency of gastrointestinal polypoid lesions in the Albanian population stressed the absence of a screening programme. According to the

Table 3 Distribution of colorectal cancer screening programmes in EU-19 as of December 2015

Country	Program			Test	Screening interval (yr)	Age (yr)	Program start	Pop target	Level of participation (%)
	Type	Status	Region						
Federation of Bosnia and Heregovina[22]	Spontaneous Organised	NatW	All country	FOBT	-	> 50	-	-	-
Republika Srpska[22]	Spontaneous	NatW	All country	FOBT	-	> 50	-	-	-
Georgia[51]	Organised	NatW	Tblisi	gFOBT	2	50-69	-	25388	53
			OutsideTblisi	gFOBT	2	50-69	-	71364	84
Iceland[18]	Programmed as organised	NatW	All country	FOBT	2	55-75[19]	-	86000[19]	
Iceland[19]	Spontaneous	NatW	All country	Colonoscopy		50-59			30
Monaco[35]	PB	Natw	All country	iFOBT (from 2015) gFOBT	2	50-80	2006	16000[35]	60
Montenegro[22,28]	None	-	-	-	-	-	-	-	-
Montenegro[28]	Not PB	PilotStudy	Danilograv, municipality of Podgorica	iFOBT	-	50-74	2010-2011	4500	33.3
Norway[20]	PB	Pilot Study	Østfold, Akershus and Buskerud	iFOBT	2		2012		
Norway[21]	PB	Pilot Study RCT	Oslo and Telemark in 1999-2001 NORCAPP	FOBT and FOBT + Sigmoidoscopy	-	55-64	1999-2000	13823	64.8
Russian Fed[42]	PB	Pilot Study	Sant Petersburb, all 18 town district	iFOBT		48-75	November 15	20000	
Russian Fed[43]	NPB	Pilot Study	Kazan, Tatarstan Republic	FOBT, DRE, questionnaire	-		2010	1071	
San Marino[36,37]	PB	Natw	All country	iFOBT	2	50-79	2009		65[37]
Serbia[31]	PB	Natw	All country	iFOBT	2	50-74		789330	58.38
Switzerland[32]	Spontaneous	NatW	All country	FOBT or Colonoscopy	2, 10	50-80	2013	13170	22
Switzerland[33]	PB	Pilot Study RCT	Glarus, Vallée du Joux Uri	FOBT and or Colonoscopy	-	50-80	2001	20000	
Switzerland[34]	PB	Pilot Study, NRCT	Vaud	iFOBT or Colonoscopy		50-69	2015		
Turkey[44]	Organised Spontaneous	NatW		FOBT		50-69	2009	11681513	30[44]
Ukraine[46,54]	PB	NatW		Not available	Not available	Not available	2002-2006		Not available

gFOBT: Guaiac test; iFOBT: Immunological test; FOBT: Not specified if gFOBT or iFOBT; DRE: Digital rectal exam; NPB: Not population-based.

WHO report[24], neither the FOBT nor colonoscopy are available at the level of public primary health care (Table 4).

Bosnia and Herzegovina, Republika Srpska: In the Federation of Bosnia and Herzegovina the incidence of CRC is 20.7 among men and 13.3 among women, with a mortality rate of 12.7 in men and 7.7 in women. Data quality is not excellent (Table 2). According to Giordano *et al*[22], spontaneous and organised screening based on the FOBT is available for those aged more than 50 years. However, Buturovic reports that in the Konjic area colonoscopy is not available[25]. As shown in Table 4, the WHO has no data on the availability of screening tests (FOBT, colonoscopy) at the level of public primary health care[26]. The tumour represents the third and second most common cancer and the second and third cause of death in the country (Table 4).

In the Republika Srpska only spontaneous screening is available to subjects older than 50 years[22]. Again, there is no clear information on screening programmes.

FYR Macedonia: In this country CRC incidence is moderately high in men (28.4) as well as women (20.5) (Table 1) and mortality rates of 15.5 and 10.8, respectively. Data quality is mediocre (Table 2). There seem to be no organised screening programmes, even though the iFOBT is available at the public primary health care level[27] (Table 4). CRC is the third most common tumour in the country for both sexes and the second cause of cancer death (Table 4).

Montenegro: The incidence of CRC in Montenegro is 36.2 among men and 21.1 among women, with a mortality rate of 20.7 in men and 12.0 in women. Data quality is poor (Table 2). A population-based screening programme using the iFOBT and involving subjects aged 50 to 74 years was conducted from February 2010 to March 2011 in Danilograv municipality (Podgorica)[28], while neither organised nor opportunistic screening is available in the other areas[22]. According

Table 4 National cancer profiles

Country	Income[1]	Total population	Cancer registry	Availability at public primary health care levels of early detection tests		Ranking CRC incidence[2]		Ranking CRC mortality[2]	
				Faecal occult blood test	Bowel cancer screening by exam or colonoscopy	Man	Woman	Man	Woman
North Europe									
Iceland[2]	High	326000	National, population-based	-	-	3rd	3rd	2nd	4th
Norway[2]	High	4994000	National, population-based	Yes	-	2nd	2nd	3rd	2nd
Central Europe									
Liechtenstein[3]	High non OECD	36925	NA	-	-	2nd	3rd	NR	NR
Monaco	High non OECD	38000	Hospital-based	Yes	Yes	NR	NR	NR	NR
Switzerland[3]	High	7997000	Sub-national population-based[a]	Yes	Yes	2nd	2nd	3rd	3rd
South Europe									
Andorra	High non OECD	78000	Hospital-based	Yes	Yes	NR	NR	NR	NR
San Marino	High non OECD	31000	National population-based	Yes	Yes	NR	NR	NR	NR
Balcanian countries									
Albania[3]	Upper middle	3162000	Sub-national, hospital-based	-	-	> 5th	5th	> 5th	5th
Bosnia and Herzegovina[3]	Upper middle	3834000	NA	-	-	3rd	2nd	2nd	3rd
Montenegro[3]	Upper middle	621000	NA	-	-	2nd	2nd	2nd	3rd
Serbia[3]	Upper middle	9553000	Sub-national	Yes	Yes	2nd	2nd	2nd	3rd
FYR of Macedonia[3]	Upper middle	2106000	National	Yes	-	3rd	3rd	2nd	2nd
Eastern Europe									
Republic of Moldova[3]	Lower middle	3514000	National, hospital-based	Yes	Yes	2nd	2nd	2nd	2nd
Russian Fed[3]	High non OECD	143000000	Sub-national, population-based[b]	Yes	Yes	3rd	2nd	3rd	2nd
Turkey[3]	Upper middle	73997000	Sub-national population-based[c]	Yes	Yes	4th	3rd	4th	3rd
Ukraine[3]	Lower middle	45530000	National, population-based	-	-	2nd	2nd	2nd	2nd
Caucasican countries									
Armenia[3]	Lower middle	2969000	National, hospital-based	Yes	-	5th	3rd	4th	2nd
Azerbaijan[3]	Upper middle	9309000	NA	Yes	-	4th	4th	5th	5th
Georgia[3]	Lower middle	4358000	Sub-national population-based	-	-	5th	> 5th	5th	> 5th

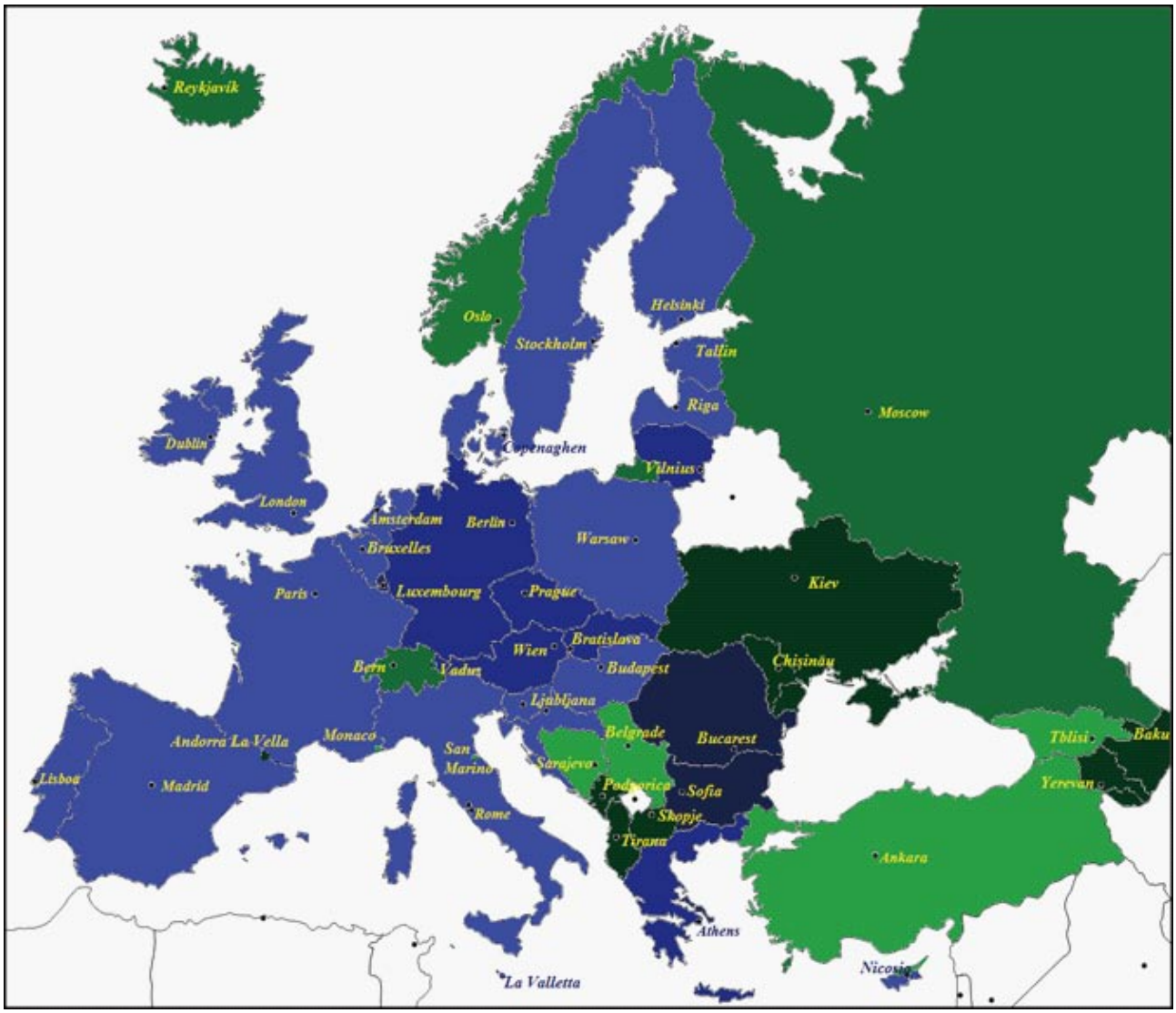
OECD (Organization for Economic Co-operation and Development): [1]It referred to pro capita Gross National Income (current US$) as indicated by World Bank: High income $382742014; High Income non OECD: $18939; Upper middle $79012014; Lower middle $2012; [2]Incidence and mortality between different cancers for country: data from http://www.who.int/cancer/country-profiles/en/adapted for each country; [3]Data available from: http://assets.krebsliga.ch/downloads/fl2014.pdf; [a]Registry in Zurich, Vaud, Valais, Ticino, St Gall-Appenzell, Neuchâtel, Graubünden and Glarus, Geneva, Basel; [b]Registry in Saint Petersburg; [c]Registry in Trabzon, Izmir, Erdine, Antalya. Information about registry existence are available from Ref. [46]. NA: Not available; NR: Not reported; CRC: Colorectal cancer.

to WHO data (Table 4), early detection tests are not available at the public primary health care level[29]. CRC ranks respectively as the second and third cause of cancer death in Montenegro (Table 4).

Serbia: At 43.3 in men and 23.3 in women, the incidence of CRC in Serbia is fairly high and the tumour is the second most common malignancy in both sexes. The mortality rates are 28.8 in men and 11.5 in women, and CRC is respectively the second and third cause of cancer death in the country. Data quality is good (Table 2). In 2013 Serbia implemented a national screening programme by extending a programmes that had been active in Vozdovac, Subotica and Zrenjanin since 2005[30]. The current programme is offered to 50 to 74 year olds without evidence of CRC and uses the iFOBT. Its results are available online. The rate of participation as of 30 September 2015 was 58.38%[31].

Central Europe

France, Poland, Hungary, and the Netherlands offer national organised programmes, and Belgium a regional programme. Austria, Germany, the Czech Republic, Slovakia, and Luxembourg provide for spontaneous screening (Figure 1). The other countries



EU-28 Countries with organised screening

	ASW-R	Inc.	Mort.
Belgium[r]	36.7	11.8
Croatia	32.9	18.7
Cyprus	24.5	6.9
Denmark	40.5	14.5
Estonia	27.2	12.3
Finland	23.5	8.3
France	30.0	10.2
Hungary	42.3	20.8
Ireland	34.9	12.2
Italy	33.9	10.8
Latvia	23.7	19.9
Malta	31.9	12.2
Netherlands	40.2	13.4
Poland	27.0	14.5
Portugal[r]	31.7	13.6
Slovenia	37.0	16.2
Spain	33.1	12.3
Sweden[r]	29.2	10.9
United Kingdom	30.2	10.7

EU-28 Countries with spontaneous screening

	ASW-R	Inc.	Mort.
Austria	26.0	9.9
Czech Republic	38.9	15.4
Luxembourg	31.5	11.2
Lithuania	23.4	13.7
Germany	30.9	10.4
Greece	13.5	7.5
Slovakia	42.7	18.0

EU-28 Countries with unknown screening status

	ASW-R	Inc.	Mort.
Bulgaria	31.5	16.0
Romania	26.4	13.4

EU-19 Country with spontaneous screening

	ASW-R	Inc.	Mort.
Iceland	28.4	7.4
Russia	24.5	15.2
Switzerland	29.4	9.3

EU-19 Countries with organised screening

	ASW-R	Inc.	Mort.
Bosnia[r]	16.6	9.8
Georgia	8.5	4.6
Monaco	NA	NA
Norway[r]	38.9	13.0
San marino	NA	NA
Sebia	32.6	16.6
Turkey[r]	16.6	10.0

EU-19 Countries with unknown screening status

	ASW-R	Inc.	Mort.
Albania	8.4	4.4
Andorra	NA	NA
Armenia	19.3	11.1
Azerbaj	6.7	4.1
Liechtstenstein	NA	NA
Monteneg	28.2	15.9
Macedonia	24.3	13.0
Moldova	28.3	16.5
Ukraine	23.4	13.7

Figure 1 Comparison between EU-28 and EU-19 according to distribution of screening programmes. Inc: Incidence; Mort:. Mortality; [r]: Regional screening; NA: Not available.

in the area are Switzerland, Liechtenstein, and the Principality of Monaco.

Switzerland: The incidence rate of CRC is 36.3 in men and 23.3 in women; CRC is the second most common neoplasm in the country (Table 4). With a mortality rate of 28.8 in men and 6.4 in women, CRC ranks as the third cause of cancer death in both sexes (Table 4). Data quality is good and in line with that of neighbouring countries (Italy, France, and Germany). Since 2002, the Swiss Federal Statistical Office has been conducting a telephone survey, the Swiss Health Interview Survey (SHIS). In 2007, it assessed for the first time the date and reason for the use of the iFOBT and/or colonoscopy and asked detailed questions on screening[32]. The 2007 results found a rate of participation of 18.9% for both methods. In 2012 participation rose to 22.2% (P = 0.036)[33]; colonoscopy rose from 8.2% in 2007 to 15% (P < 0.001) and the iFOBT fell from 13% to 9.8% (P = 0.002). In 2007 the prevalence of CRC screening among respondents was 24.5% among higher-income respondents (> $6000 a month) and 10.5% in those with a low income (< $2000); the 2012 survey found a similar difference (respectively 28.6% and 16.0%). There was no association with education or occupation[32]. Since 1 July 2013 the test (colonoscopy every 10 years and iFOBT every 2 years) is partially covered by the mandatory insurance for those aged 50 to 69 years. In Uri Canton an organised programme is offered to 50-80 year olds without a past or current history of CRC. The programme was introduced in 2000 and the results of the first round have been published[33]; the patient could choose among colonoscopy, sigmoidoscopy, and iFOBT + sigmoidoscopy, and more than 70% opted for colonoscopy. Another programme in Vaud Canton offers screening to individuals aged 50 to 69 years having no risk factors or a past or current history of CRC; they can choose between the iFOBT every 2 years and colonoscopy every 10 years; the excess is paid for by the Cantonal administration, the remaining expenses are sustained by the patient. The programme is co-ordinated by the Fondation Vaudoise pour le Dépistage du Cancer[34].

Liechtenstein: In this tiny state the incidence data are provided by the National Bureau of Statistics. CRC is the second most common cancer in men and the third in women. No data are available on CRC screening programmes, neither through institutional websites nor through the WHO (Table 4).

Principality of Monaco: Official incidence and mortality data validated by the WHO are not available for this city-state, but they are probably similar to those of France. The Centre Monégasque de Dépistage has been coordinating the CRC screening campaign since 2006. The programme uses the iFOBT. Subjects receiving a letter of invitation can choose between picking up the examination kit at their general practitioner (GP) or at the screening centre. Those with a positive test are referred to the Centre Hospitalier Princesse Grace, where digestive endoscopy is performed to establish the cause of the bleeding. The service is offered to residents and foreign workers aged 50-80 years. Participation is about 60%. In March every year, the Blue March is organised in France and the Principality to promote CRC awareness and focus the attention of the population on the value of CRC prevention[35].

Southern Europe

In this region, Italy, Spain, Malta, and Cyprus offer national organised programmes, and Portugal a regional organised programme. In Greece screening is spontaneous (Figure 1). There are also two tiny states, Andorra and San Marino.

San Marino: Official incidence and mortality data are not available; however, since San Marino is nestled in the Italian region of Emilia-Romagna, they should be similar to those of Italy. A national programme, offered to individuals aged 50 to 75 years and managed by the Screening Centre of the Istituto per la Sicurezza Sociale, has been in place since 2009. The test is delivered home by mail and the recipient is asked to take it to the relevant Health Centre. Failure to do so in three months results in a reminder. Subjects with a positive test are referred to the State Hospital for a second-level examination, usually colonoscopy[36]. Participation rates (65%) have been illustrated at a press conference and are encouraging[37].

Andorra: WHO incidence and mortality data are not available, but they can reasonably be considered to resemble those of Cataluña. According to the 2014 WHO report - Cancer Country Profiles, CRC screening with the iFOBT and colonoscopy are generally available at the public primary health care level in this small Pyrenean state[38] (Table 4). However, unlike the case of breast cancer, the institutional website provides no information on CRC prevention.

Eastern Europe

In this area, Romania and Bulgaria do not offer organised screening (Figure 1). The other states in the region include the Republic of Moldova, the Russian Federation, Turkey and Ukraine.

Republic of Moldova: In this country CRC is the second most common neoplasm in men and women alike (Table 4) with an incidence of 36.0 and 23.0 respectively. The mortality rate is 22.0 and 12.6, respectively; CRC is the second cause of cancer mortality in the country (Table 4). The literature provides

no information on screening programmes, but the FOBT and colonoscopy are both available at the public primary health care level[39] (Table 4).

Russian Federation: CRC is the third tumour by incidence and mortality among men (respectively 30.0 and 19.9) and the second among women (respectively 21.8 and 11.5) (Tables 1 and 4). Data quality is mediocre (Table 2). No national screening programmes are in place[40]. The poor awareness regarding CRC involves a high rate of late diagnoses (25.6% in stage Ⅳ *vs* 18.8% in the United States) despite the fact that the iFOBT and colonoscopy are largely available at the public primary health care level[41] (Table 4). A screening campaign launched in November 2015 in the Saint Petersburg area, which hosts the sole cancer registry in the Federation[42], follows an earlier, small-scale programme set up in the Kazan region[43] (Table 3).

Turkey: In Turkey CRC incidence is 20.6 and 13.1 in men and women, respectively (Table 1). The mortality rate is 12.6 and 10.8, respectively (Table 1). Data quality is mediocre (Table 2). The national Cancer Control Department has been promoting cancer prevention campaigns since 2003. In 2009 there were no active population-based programmes, but merely some sporadic pilot studies[44]. However, a KETEM centre per province (including 2 in Istanbul and 3 in Ankara) supervise the execution of cancer screening (breast and cervical cancer) according with national guidelines; CRC was added in 2009. Screening is not covered by Social Security provisions and is sustained by the Health Ministry only for older and poorer people. Screening is largely spontaneous, but some centres like Ankara have started population-based programmes[44] (Table 3). In the future, GPs will be given the task of encouraging patients to pursue early CRC detection[44]. Both the FOBT and colonoscopy are available at the public primary health care level, as reported by the WHO country cancer profile[45]. CRC is the third and fourth cause of cancer death in the country (Table 4).

Ukraine: In Ukraine, the incidence rate of CRC is 29.9 in men and 19.9 in women and the tumour ranks second as a cause of cancer death. The mortality rate is 18.8 in men and 10.8 in women. Data quality is excellent (Table 2). According to the cancer registry, the 2002-2006 cancer plan included prevention programmes for cervical, breast, colorectum, prostate, skin, and oral cavity[46]. Based on WHO data, screening tests are not available at the public primary health care level[47] (Table 4).

Caucasian countries

These states lie on the extreme eastern boundary of Europe, where the Caucasus traditionally represents the geographical border with the Middle East.

Armenia: In Armenia CRC is the fifth most common tumour among men (22.8) and the third among women (17.0); it is the fourth cause of cancer death among men (13.4) and the second among women (9.7) (Tables 1 and 4). Data quality is fairly poor (Table 2). The literature supplies no information on screening, but the FOBT is available at the level of public primary health care[48].

Azerbaijan: In Azerbaijan CRC incidence is fairly low in both sexes (7.1 in men and 6.4 in women) and is the fourth most common tumour. CRC mortality is lower in men (4.3) than in women (19.8) and the tumour is the fifth cause of cancer death in the country (Tables 1 and 4). Data quality is poor (Table 2). Although there are no published data on screening, the WHO report indicates that the FOBT is available at the public primary health care level[49].

Georgia: In this country CRC incidence is low: 9.9 in men and 7.5 in women. The mortality figures are 5.5 in men and 4.0 in women. Data quality is poor (Table 2). Even though according to the WHO reports neither the FOBT nor colonoscopy is available for first-level screening[50], the literature and the official websites mention a CRC screening campaign and report its results[51]. These data are summarised in Table 2.

DISCUSSION

Improvements in the health status of populations and the progressive increase in life expectancy call for the promotion and diffusion of healthy lifestyles, to reduce the impact of non-communicable diseases; in particular, cancer entails an extremely high social and psychological burden. The chief mission of the Council of Europe is to promote and protect human rights in member states by issuing orientation papers and guidelines. In particular, art. 11 of the Social Charter, "The Right to Protection of Health", explicitly mentions the promotion of the health of the citizens of member states[52] and art. 3 of the Convention on Human Rights and Biomedicine makes reference to equal and appropriate access to health care[53].

Interestingly, comparison of neighbouring countries offering regional screening shows, for instance, that incidence and mortality rates are 38.9 and 13.0 in Norway and 29.2 and 10.9 in Sweden, whereas in Finland, where a national organised programme is available, they are respectively 23.5 and 9.3[54].

Epidemiological data quality varies broadly between EU-28 and EU-19 countries. In terms of incidence, only 30% of EU-19 countries rank high in data quality (A, B and C), as opposed to 86% of EU-28 states. The same applies to mortality data: 52%of EU-19 countries as against all EU-28 countries are found in the high ranks

(1, 2, or 3) (Table 2).

Assessment of the method of collection of incidence data showed that only 32% of EU-19 countries were found in the top three quality classes (A, B and C) as against 89% of EU-28 countries. For the mortality data, 63% of EU-19 countries were found in the highest ranks as opposed to all EU-28 member states (Table 2). The continuous improvement in the quality of epidemiological data collection critically supports public health decision-making, in that it represents the phenomena studied in an increasingly accurate manner in all geographical areas. This is all the more important at a time when the European continent is besieged by problems such as the economic downturn, climate change, international tensions and, last but definitely not least, the management of strong migration flows. The economic crisis has hampered the activation of large-scale screening programmes in countries, like Iceland[19], where the recession has had a devastating impact, involving their postponement. In other emerging states and areas, like Serbia[30,31], Georgia[51], and Saint Petersburg[42], population-based programmes have been implemented despite the crisis, making cancer prevention a priority, also to reduce social inequality. Clearly, it is critical to stimulate awareness of the importance of cancer prevention through screening.

In a recent British study, Moffat *et al*[55] found that awareness is crucial where cancer and its early detection are concerned. The authors measured CRC symptom knowledge before and after an awareness campaign directed at lower-income subjects, and showed that the campaign improved the knowledge of suspicious symptoms. Notably, the campaign also increased screening requests to GPs. Another key finding was a greater awareness of CRC among the elderly, suggesting that differential campaigns are not required for different age classes. Large-scale campaigns like the Blue March, organised in France and Monaco Principality, should therefore be encouraged[35].

The absence of organised screening increases the social burden of cancer, delaying the adoption of treatments of proven efficacy that induce as little disability as possible. The situation is especially clear in economically and technologically advanced countries like Switzerland, where a significantly lower demand for testing has been found among low-income than high-income citizens. Conceivably, the problem is even more severe in countries where early detection testing is not provided by the national health care system and is predominantly out of pocket, compounding the vulnerability of the poorer groups in the population. State funding is a problem in several countries, like Turkey, where dedicated cancer screening centres are found in each province and are theoretically easier to reach. In fact, however, the fact that most people have to pay for their tests, since only the poorest and oldest benefit from state help, prevents access by large swathes of the population. It should be stressed that the activation of organised screening programmes is not a net cost to emerging countries[56], but is actually cost-effective[57,58], also considering that early tumour detection considerably reduces subsequent social and health costs[43].

The situation of these countries contrasts with the one characterising small states like Monaco Principality and San Marino, where the tiny population enables a more effective organisation of health care, including prevention. Monaco Principality deserves high praise for extending screening benefits to foreign workers[35].

Screening professionals and activity

Finally, gastroenterologists play an important role in CRC screening. They should not view early detection testing as a practice undermining their expertise, but as a resource that adds to their specialist training[59]. The same applies to clinical pathologists: in some countries, like Russia, their training is predominantly oriented to necroscopy[40]: diagnosing living patients is therefore a challenge for all health care figures. The epidemiologist also has a critical role in local screening co-ordination, patient flow organisation, data management, and data transmission to cancer registries, to improve cancer knowledge and treatment in the various districts. GPs are an essential link in the prevention chain, since they have the task of raising the awareness of those who are at risk and of stimulating those who would benefit from screening to undergo testing, without alarming them. In Turkey, for instance, GPs have the task of directing patients to screening[44]. Patient associations and health care professionals should also work to increase the awareness of institutions and law-makers towards screening and its benefits.

In conclusion, cancer screening should be viewed as a key health care tool, also because investing in screening protects the weakest in the population, decreases the social burden of cancer, and reduces all types of health care costs, including those for radical surgery, long-term hospitalisation, and chemotherapy. Finally, screening for those at risk should be stimulated, offered without charge, and adequately publicised irrespective of the health care system organisation in place.

COMMENTS

Background

Although colorectal cancer (CRC) is a tumour for which screening has proven efficacy and cost-effectiveness, in several European countries screening implementation is fraught with difficulties. CRC incidence is quite variable among European countries, and the lower rates found in eastern Europe are higher than the world mean.

Research frontiers

The Council of Europe has recommended the priority activation of CRC screening programmes. According to a 2008 European Commission report on the diffusion of CRC screening programmes in the EU, only 12 of the then 22

member states had population-based screening programmes; the others were recommended to provide to their citizens equal access to cancer prevention.

Innovations and breakthroughs

The present paper provides a systematic review of the screening programmes that are active in the non-EU 28 members of the Council of Europe, using data collected from institutional websites and from the literature. Besides reviewing the epidemiological data (incidence, 5-year prevalence, and mortality), it undertakes a critical examination of their quality and provides key information on colorectal cancer and the prevention strategies adopted in each country.

Applications

The absence of organised screening increases the social burden of cancer, delaying the adoption of treatments of proven efficacy that induce as little disability as possible. Conceivably, the problem is even more severe in countries where early detection testing is not provided by the national health care service and is predominantly out of pocket, compounding the vulnerability of the poorer groups in the population. Notably, the activation of organised screening programmes in emerging countries would actually be cost-effective, also considering that early tumour detection considerably reduces subsequent social and health costs.

Terminology

Fecal occult blood (FOB) refers to blood in the feces that is not visibly apparent. A fecal occult blood test (FOBT) checks for hidden (occult) blood in the stool (feces); immunochemical test (iFOBT) is based on human hemoglobin antibodies.

Peer-review

This review paper covers recent topics in CRC screening, and is concisely written. The information given is helpful to promote the further advance in the field.

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CASE REPORT

Idiopathic abdominal cocoon syndrome with unilateral abdominal cryptorchidism and greater omentum hypoplasia in a young case of small bowel obstruction

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Abstract

Abdominal cocoon syndrome (ACS) is a rare cause of intestinal obstruction due to total or partial encapsulation of the small intestine by a fibrocollagenous membrane. Idiopathic ACS with abdominal cryptorchidism and greater omentum hypoplasia is even rarer clinically. We successfully treated a 26-year-old male case of small bowel obstruction with acute peritonitis. He was finally diagnosed with idiopathic ACS with unilateral abdominal cryptorchidism and greater omentum hypoplasia during exploratory laparotomy. He then underwent enterolysis, cryptorchidectomy, and appendectomy. He recovered gradually from the operations and early postoperative inflammatory ileus. There has been no recurrence of intestinal obstruction since the operation, and he is still in follow-up. We analyzed his clinical data and retrospectively reviewed the literature, and our findings may be helpful for the clinical diagnosis and treatment on ACS.

Key words: Abdominal cocoon syndrome; Abdominal cryptorchidism; Intestinal obstruction; Diagnosis; Treatment

Core tip: Abdominal cocoon syndrome (ACS) is a rare abdominal disease where a portion or all of the abdominal organs are wrapped in a dense membrane-like fibrous tissue. Intestinal obstruction is the main

clinical manifestation of ACS. Because of its rarity and lack of characteristic symptoms, ACS is fairly difficult to diagnose pre-operatively. Surgeons should be aware of this disease when confronted with a case of intestinal obstruction whose abdominal radiography shows intestinal loop aggregation cluster. Accompanying cryptorchidism is possible, and a careful physical examination and operative exploration for the undescended testicle should be performed. Postoperative care and dietary guidance are very important to the rehabilitation of ACS patients. Postoperative re-adhesion and early postoperative inflammatory ileus easily occur after extensive enterolysis.

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INTRODUCTION

Abdominal cocoon syndrome (ACS) is a rare abdominal disease where a portion or all of the abdominal organs are wrapped in dense membrane-like fibrous tissue. It was first reported in 1978 and is also known as idiopathic sclerosing peritonitis, primary sclerosing peritonitis, and sclerosing encapsulating peritonitis[1,2]. The etiology and epidemiological characteristics of idiopathic ACS remain unknown. Intestinal obstruction is the main clinical manifestation of ACS. Usually, ACS is chanced upon during abdominal surgery. Because of its rarity and lack of characteristic symptoms, ACS is fairly difficult to diagnose prior to the operation.

We successfully treated a young male with intestinal obstruction secondary to idiopathic ACS with unilateral abdominal cryptorchidism and greater omentum hypoplasia.

CASE REPORT

A 26-year-old male patient was admitted to the emergency department with complaints of abdominal pain, nausea, and vomiting for about 10 h on November 25, 2014. Although this patient had a history of overeating before hospitalization, he did not have a history of chronic systemic disease or abdominal trauma. Upon physical examination, there was asymmetrical distension and general tenderness with heightened intestinal sounds, especially prominent in the right middle abdomen. The right testicle was not palpable in the scrotum. The laboratory examinations showed normal peripheral leukocyte counts (7.98 × 10^9/L), but the ratio of neutrophils was slightly elevated to 75.1%. Abdominal radiography detected the dilated intestine with air-fluid levels in the right middle abdomen (Figure 1). Abdominal computed tomography detected dilated small intestinal loops containing air-fluid levels clustered in the middle abdomen that were surrounded by a thick and sac-like membrane (Figure 2). The widespread adhesions between the peritoneum and small intestine were found during exploratory laparotomy. With further exploration, a cocoon-like fibrous structure was identified in the middle abdomen that surrounded the majority of small intestine (Figure 3). The right undescended testicle had softened and adhered tightly to the fibrous membrane near the appendix. The greater omentum was hypoplastic. When the cocoon-like fibrous membrane was opened, the small intestinal segments were dilated due to obstruction, but they were otherwise normal in structure. The obstruction was caused by fibrous bands of irregular thickness inside the cocoon-like fibrous membrane. The operation was completed after total excision of the fibrous membrane and removal of the adhesions. The released small intestinal segments were rearranged and coated with sodium hyaluronate. At the same time, the right undescended testicle and appendix were resected. Postoperative pathologic examination showed that the testis with interstitial fibrosis had no spermatogonium, primary spermatocyte, secondary spermatocyte, spermatid, or spermatozoon present in the seminiferous tubules (Figure 4).

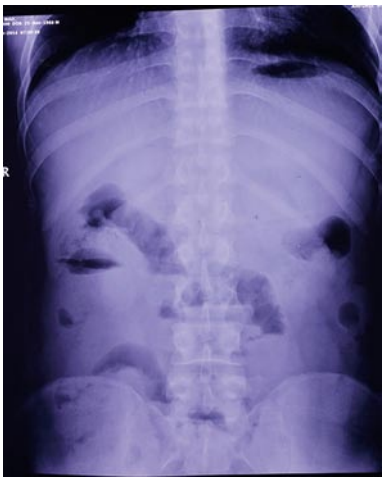

Figure 1 Abdominal radiography. The dilated intestine with air-fluid levels was prominent in the right middle abdomen.

Patient recovery during postoperative week one went well, but he suffered early postoperative inflammatory ileus (EPII) performance with intermittent abdominal pain and vomiting on postoperative day 12. After a series of symptomatic treatments for about 5 d, including fasting, gastrointestinal decompression, inhibiting secretion of digestive juices by octreotide, nutritional support, and maintaining balance of electrolytes; he recovered gradually and was discharged on December

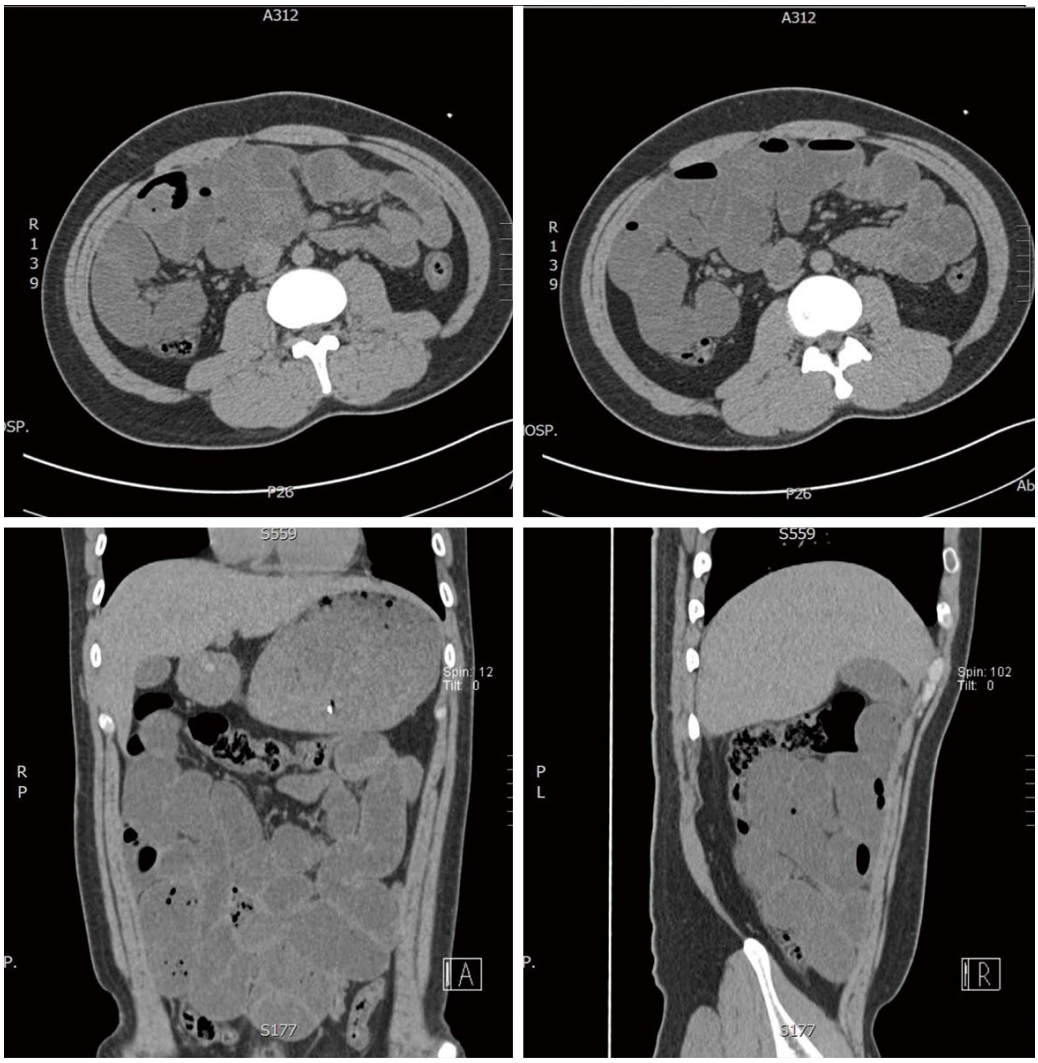

Figure 2 Abdominal computed tomography scans. Dilated small intestinal loops containing air-fluid levels were clustered in the right middle abdomen and surrounded by a sac-like membrane.

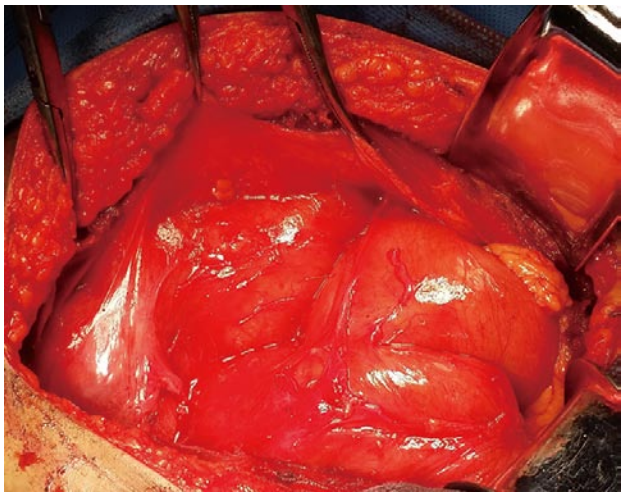

Figure 3 Intraoperative findings. Dilated small intestine was surrounded by a capsular structure in the right middle abdomen, which had a regular surface composed of natural fibrous membranes.

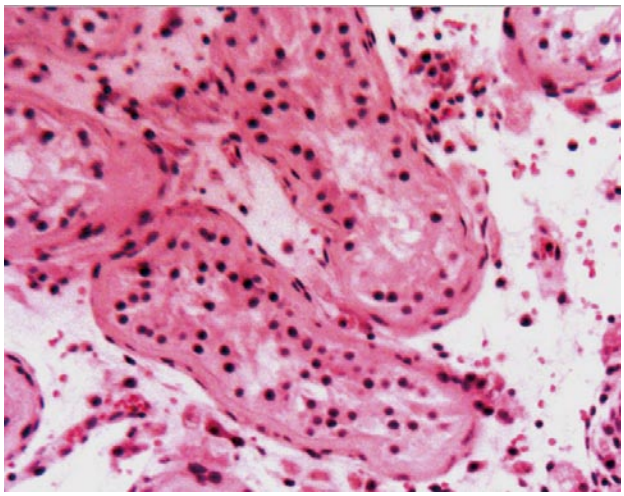

Figure 4 Pathologic examination (HE × 200). The testis with interstitial fibrosis had no spermatogonium, primary spermatocyte, secondary spermatocyte, spermatid, or spermatozoon in the seminiferous tubules.

20 2014. The intestinal obstruction has not recurred, and he is still undergoing follow-up.

DISCUSSION

According to whether its etiology is explicit or not, ACS can be divided into two subtypes: primary (idiopathic) and secondary[3]. Usually, secondary ACS results in chronic asymptomatic peritonitis, such as endometriosis, retrograde menstruation, peritoneal dialysis, and abdominal tuberculosis[4]. Although idiopathic ACS is rare clinically, patients with idiopathic ACS are often accompanied by the absence of the greater omentum and cryptorchidism, suggesting that genetic factors may play a role in the etiology of idiopathic ACS. Our case developed intestinal obstruction and peritonitis without any other known risk factors; but the typical fibrous membrane surrounding the small intestine with greater omentum hypoplasia and undescended testicle confirmed that the diagnosis of idiopathic ACS is correct.

Although ACS is a rare cause of intestinal obstruction, we should vigilantly remember this disease when faced with a case of intestinal obstruction whose abdominal radiography shows intestinal loop aggregation into a cluster[5,6]. When ACS is suspected, accompanying cryptorchidism should be suspected, and a careful physical examination and operative exploration for the undescended testicle should be performed. How to deal with the undescended testicle remains controversial[7,8]. Because surgical options of the undescended testicle interfere with patient fertility and have a high risk of seminoma, it is necessary to fully communicate with the patient about this matter before the operation is performed. As for the appendix, we think appendectomy may be a good option for patients with ACS, because the occurrence of postoperative abdominal adhesions is inevitable, and it will be very difficult to perform appendectomy in these postoperative patients if appendicitis or other appendical diseases occurs[9]. The surgeon should pay attention to reserve the vital intestine and possibly the ileocecal valve and to avoid resecting the "cocoon", as tumor results in short bowel syndrome.

As to the patients with ACS, postoperative care and dietary guidance are very important to their rehabilitation. Postoperative re-adhesion and EPII occur easily after the extensive dissection of the enterolysis[10]. Therefore, we should encourage patients to get out of bed early in order to promote intestinal peristalsis recovery and to avoid recurrence of intestinal obstruction. The patients' diet should be gradually restored since their gastrointestinal function recovery is manifested as exhaust and defecation. The whole process of diet restoring maybe take about 10 d to 2 wk, beginning from liquid diet and progressing to semi-liquid diet, soft diet, and finally to general diet. Our case's discharge time was delayed for about 7 d by EPII because of improper self-eating.

COMMENTS

Case characteristics

Patient was admitted to the emergency department with complaints of abdominal pain, nausea, and vomiting for about 10 h.

Clinical diagnosis

Upon physical examination, there was asymmetrical distension and general tenderness with heightened intestinal sounds, which were especially prominent in the right middle abdomen.

Differential diagnosis

Abdominal computed tomography detected dilated small intestinal loops containing air-fluid levels clustered in the middle abdomen that were surrounded by a thick and sac-like membrane.

Laboratory diagnosis

During exploratory surgery, a cocoon-like fibrous structure surrounding the majority of the small intestine was identified in the middle abdomen.

Imaging diagnosis

Abdominal computed tomography detected dilated small intestinal loops containing air-fluid levels clustered in the middle abdomen that were surrounded by a thick and sac-like membrane.

Pathological diagnosis

Pathologic examination showed that the testis with interstitial fibrosis had no spermatogonium, primary spermatocyte, secondary spermatocyte, spermatid, or spermatozoon in the seminiferous tubules.

Treatment

The patient underwent a series of operations, firstly exploratory laparotomy, and then enterolysis, cryptorchidectomy, and appendectomy.

Term explanation

Abdominal cocoon syndrome (ACS) is a rare cause of intestinal obstruction due to total or partial encapsulation of the small intestine by a fibrocollagenous membrane.

Experiences and lessons

When encountering a suspected case of ACS, the surgeon must be aware of the possibility of accompanying cryptorchidism. Then, a careful physical examination and operative exploration for the undescended testicle should be performed. Postoperative care and dietary guidance are very important to their rehabilitation.

Peer-review

This manuscript is a very interesting case report of a rare disease, ACS. Here, the patient presented with a small bowel obstruction with acute peritonitis. ACS is a rare disease where a portion of or all of the abdominal organs are wrapped in dense membrane-like fibrous tissue.

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EDITORIAL

Surgical dilemmas in the management of colorectal liver metastases: The role of timing

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Abstract

Colorectal cancer (CRC) is an emerging health problem in the Western World both for its raising tendency as well as for its metastatic potential. Almost half of the patients with CRC will develop liver metastases during the course of their disease. The liver surgeon dealing with colorectal liver metastases faces several surgical dilemmas especially in the setting of the timing of operation. Synchronous resectable metastases should be treated prior or after induction chemotherapy? Furthermore in the case of synchronous colorectal liver metastases which organ should we first deal with, the liver or the colon? All these questions are set in the editorial and impulse for further investigation is put focusing on multidisciplinary approach and individualization of treatment modalities.

Key words: Colorectal cancer; Chemotherapy; Timing of surgery; Colorectal liver metastases; Liver first procedure; Multidisciplinary approach; Individualized treatment strategies

Core tip: The treatment of colorectal cancer with colorectal liver metastases is a challenge for the multidisciplinary medical team dealing with this problem. The timing of surgery both for synchronous as well as for metachronous metastases is always a matter of debate. Multidisciplinary approach and individualization of treatment strategies is suggested.

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Colorectal cancer (CRC) remains an important public health issue as it is the third leading cause of death for both men and women in the United States and the most frequent cause of cancer among patients aged 75 years and older[1]. Furthermore approximately 10%-25% of patients with CRC present at the time

of diagnosis with liver metastases (CRLM) while 25% will develop liver metastases in the future, a fact that means almost half of the patients with CRC will develop liver metastatic disease. While in the past the presence of multiple or enlarged CRLM was a sign towards palliative treatment the progress in liver surgery, medical oncology and interventional radiology has allowed us to perform liver resections for colorectal liver metastases with intention to treat[1].

The surgeon who confronts with colorectal liver metastases faces several problems. Should I operate first or is it better for the patient to receive neoadjuvant treatment. And when it comes to the operation should I operate both liver and colon or separately and if separately which organ first. The conventional way of thinking in patients with resectable synchronous colorectal liver metastases is to offer an upfront operation and the reason for this attitude is the fear that CRLM will not respond to chemotherapy and that during the time of chemotherapy the liver tumors will grow and become unresectable so that the patient will lose the possibility of a curative liver operation[2]. Though, in this case scenario there is always a possibility to develop post-operative complications that will delay the chemotherapeutical approach and the patient will not benefit from medical oncology. Furthermore if we choose chemotherapy prior to surgical resection the tumor load within the liver is assumed to decrease and therefore we could achieve a higher percentage of complete resection rate (R0 resection) as well as the ability to perform minor hepatectomies, another argument counting for neoadjuvant chemotherapy. In some cases we might as well have a complete response, a phenomenon called "vanishing metastases" in the pertinent literature, "where the dream of the oncologist becomes the nightmare of the surgeon", because even if we have a complete radiological response there are still some active tumor cells that require surgical resection. In order to avoid such a problem there several solutions proposed, like marking the metastatic lesions prior to oncological referral. However, the choice of chemotherapy prior to surgical resection will have favorable results only under the circumstance that the tumor is sensitive to the therapeutical regimen we choose, so a complete examination of the k-ras and b-raf should be performed. Another pitfall of this approach is the development of chemotherapy adverse effects that will delay surgery, especially if we take into consideration that almost all oncological agents develop liver toxicity (*e.g.,* blue liver)[3].

The second problem for the surgeon is whether to proceed to a combined liver and colon operation or a staged one. This problem has created a debate in the pertinent literature. The combined operation can theoretically solve the patient's surgical problem with one shot. This approach has a better impact on the patient's psychology as well as the financial aspect because we have one admission and one surgical procedure and in total decreased time of hospitalization. On the other hand a combined procedure can lead to an increased risk of adverse effects of both colectomy and hepatectomy and in that case the patient will face a delayed post-operative course and might lose the time window for chemotherapy. Most authors conclude that it would be better to avoid low anterior colectomies with major liver resections. For the rest of the cases there is no consensus and the decision should be individualized taking into consideration the tumor load, the patient's performance status and the experience of the institution[4]. Furthermore for the case the surgeon decides to proceed to a combined operation our group has published experimental data demostrating that if we prefer to start with the liver and perform an intermittent Pringle maneuver the post-operative outcomes are favorable[5].

There is also a great amount of patients who present with unresectable liver metastases or with metastatic disease to other organs apart from the liver (lungs, peritoneal deposits *etc.*) at initial diagnosis. Patients with colorectal liver metastases initially considered as unresectable should be under close follow up by the surgical team during chemotherapy. Classical therapeutical agents in combination with biological agents (monoclonal antibodies) have increased the resectability rate of these patients. Furthermore, the use of special maneuvers such as portal vein ligation or embolization together with the above mentioned chemotherapeutical agents enable the decrease of tumor load with a concomitant increase of remnant liver volume so that the number of patients who are candidates for liver resection raises even more. For patients who develop metastatic disease to other organs beside the liver the site of metastasis affects both the treatment and prognosis. In patients with colorectal metastases to liver and lung and in some cases also the peritoneum the most important factor that affects the prognosis is the resectability of the metastatic lesion or lesions, especially the liver metastases[6].

The management of a patient with synchronous colorectal liver metastases is a difficult, complicated and provocative problem to solve. With the evolution of science and technology the resectability rate has raised and more patients have favorable outcome. Surgeons are always enthusiastic but a lot of factors must be taken into consideration before choosing the best approach for each patient. The decision for the optimal treatment should be individualized, based on the results of a tumor board taking into consideration the opinion of surgeons, radiologists and oncologists. However, the most important predictive factors are the patient's performance status, the tumor load and the biological behavior of the disease.

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TOPIC HIGHLIGHT

2016 Liver Transplantation: Global view

How important is donor age in liver transplantation?

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Abstract

The age of liver donors has been increasing in the past several years because of a donor shortage. In the United States, 33% of donors are age 50 years or older, as are more than 50% in some European countries. The impact of donor age on liver transplantation (LT) has been analyzed in several studies with contradictory conclusions. Nevertheless, recent analyses of the largest databases demonstrate that having an older donor is a risk factor for graft failure. Donor age is included as a risk factor in the more relevant graft survival scores, such as the Donor Risk Index, donor age and Model for End-stage Liver Disease, Survival Outcomes Following Liver Transplantation, and the Balance of Risk. The use of old donors is related to an increased rate of biliary complications and hepatitis C virus-related graft failure. Although liver function does not seem to be significantly affected by age, the incidence of several liver diseases increases with age, and the capacity of the liver to manage or overcome liver diseases or external injuries decreases. In this paper, the importance of age in LT outcomes, the role of donor age as a risk factor, and the influence of aging on liver regeneration are reviewed.

Key words: Liver transplantation; Liver regeneration; Graft survival; Old donor; Aging

Core tip: Because of a donor shortage, the use of grafts from old donors has become widespread. Donor age is

related to worse outcomes after liver transplantation, higher rates of graft failure, biliary complications and a worse graft survival. In recipients with hepatitis C, the impact of donor age is even more evident. Aging-related changes at the hepatocellular level may contribute to a decreased capacity of the liver to manage or overcome liver diseases and injuries. This review summarizes the evidence regarding the impact of donor age on liver transplantation outcomes.

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INTRODUCTION

In the recent years a considerable change in donor age distribution of liver transplantation (LT) has been observed, as shown in the figures from the European Liver Transplant Registry (ELTR), with a rising percentage of livers proceeding from donors older than 60 years. In 1989, only 1% of livers proceeded from donors over 60 years of age. This rate escalates to 15% in 1999, 20% in 2001, and 29% in 2009[1,2]. In Spain, between 1984 and 1995, only 11.5% of donors were age 55 years or older, while between 2011 and 2012, 61.8% of donors were 55 years or older (Figure 1)[3]. The United Network for Organ Sharing (UNOS), in the United States, reports that in 1989, 2.4% of donors were age 50 years or older, but this rate increased to 29% in 1999 and to 33% in 2013 (Figure 2).

The impact of donor age on LT has been evaluated in different studies with contradictory results. Many studies did not observe differences in graft survival according to donor age[4,5], on the contrary others report an increases of complication rates and poorer survival following transplantation from older donors[6,7]. Furthermore, a relationship has been described between allografts obtained from older donors and a faster progression of fibrosis after LT in recipients infected with hepatitis C virus (HCV)[8,9].

IMPACT OF DONOR AGE ON LIVER TRANSPLANT OUTCOMES

Deceased donor liver transplant

Studies based on institutional registries have evaluated the effects of donor age on patient and graft survival in the largest patient series[1-3,6,7]. In the ELTR, the 1-year survival of patients who received transplants between 1998 and 2001 was similar for all donor age groups[2]. In a recent analysis of the same ELTR database, graft survival was significantly higher if the organs proceed

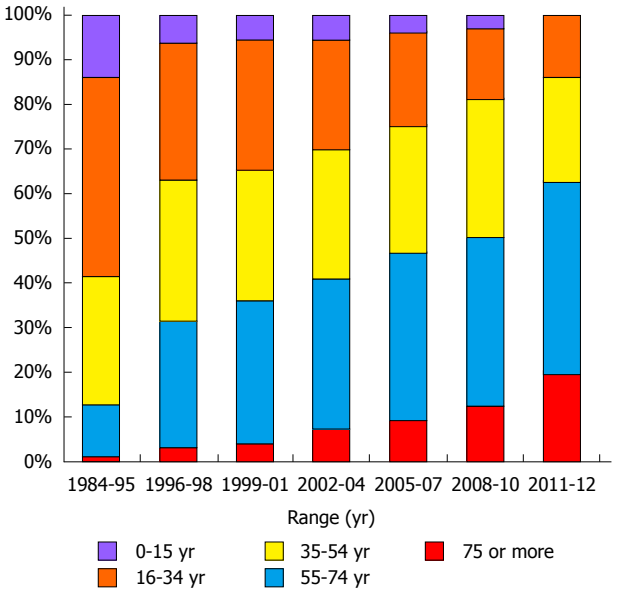

Figure 1 Change in distribution of donor age in recent years. Source: Spanish Liver Transplant Registry.

from donors younger than 55 years *vs* donors older than 65 years (65% *vs* 57%, $P < 0.0001$)[1]. An analysis of the data collected by the Spanish Registry for Liver Transplantation between 1991 and 2013 shows that donor age influences LT outcome (Figure 3). LT performed with deceased donors over age 55 years had a slight but significant worsening in actuarial graft survival one year after LT compared with those realized with graft from donors younger than 55 years. The difference in graft survival between the two groups was more evident at 5 years after LT[3]. Feng *et al*[10] recently analyzed donor risk factors in LTs finding that donor age over 60 years was the strongest risk factor for graft failure. In this analysis of the data collected from the Scientific Registry of Transplant Recipients, donor age over 40 years and especially over 60 years, donation after cardiac death, and split/partial grafts were strongly associated with graft failure. In a retrospective analysis performed using data obtained from the UNOS, Reese *et al*[11] found that performing LTs with donors who were ⩾ 45 years old increased the risk of graft failure at 90 d after transplantation. Moreover, these authors found that a combination of prolonged cold ischemia time and older donor age were associated with a decrease in graft survival after LT. We performed a prospective analysis to establish if donor age over 60 years could be a risk factor for higher incidence of complications or graft failure[12]. We did not observe differences in the initial graft function between groups. Moreover in the older donor group we did not observe any case of primary non-function and patient survival was not affected. Nevertheless, graft survival at 12 mo was decreased by about 15% in the older donor group, although patient survival was not affected.

Other studies show different results. Anderson *et*

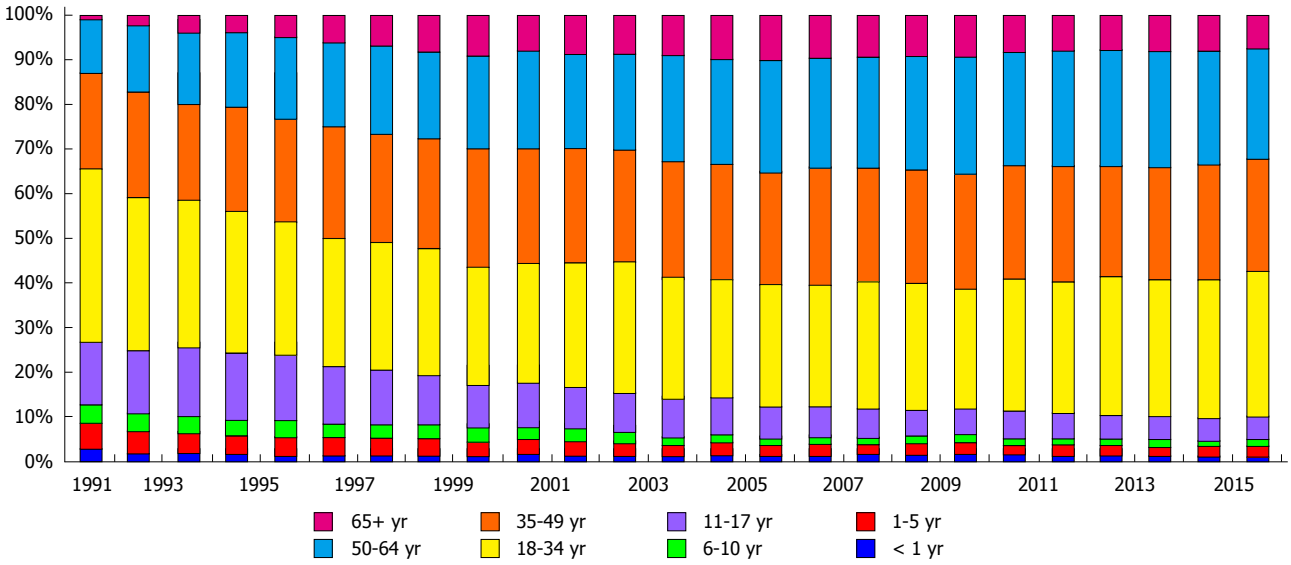

Figure 2 Change in distribution of donor age in recent years. Source: United Network for Organ Sharing reports.

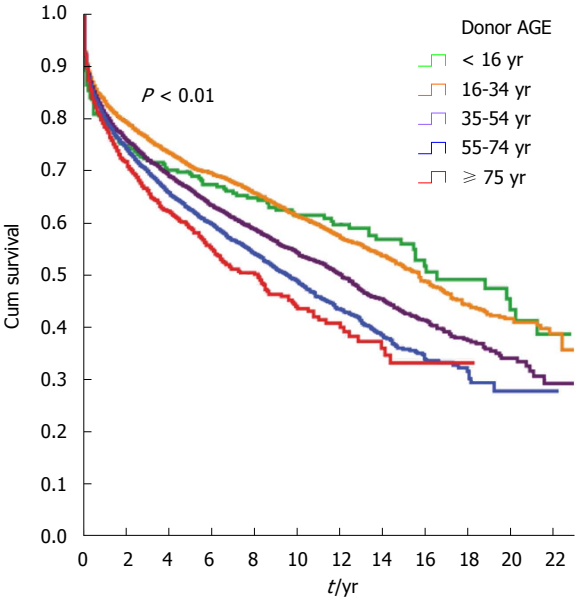

Figure 3 Graft survival depending on donor age. Source: Spanish Liver Transplant Registry.

al[13] analyzed 741 LTs performed between 1990 and 2007 and did not found significant difference in overall graft and patient survival with donors younger than 60 years compared to those aged 60 or older. However, when cases with donors ⩾ 60 years were compared with each other from different time period, the authors observed that the LT performed after 2001 had a better patient and graft survival. LT performed before 2001 had significantly longer cold ischemic times compared with those performed after 2001. From this study, these authors concluded that donor age *per se* is not a disadvantage for graft or patient survival, but that there was a possible interaction between donor age and other factors such as ischemia time.

Alamo *et al*[14] conducted a case-control single-center study and examined the outcomes of 129 livers transplanted from donors older than age 70 years. The authors observed no differences in survival but did identify a greater incidence of ascites and primary dysfunction, probably secondary to a delayed start in graft function. They recognized that recipient Model for End-Stage Liver Disease (MELD) score and cold ischemia time were parameters associated with a poor prognosis. In addition the authors found that some donor factors were associated with a poor prognosis: diabetes, hypertension, and weight greater than 90 kg. With these results, this group concluded that LT with liver grafts from elderly donors is safe but that the selection of donors and recipients must be done with care. Kim *et al*[15] retrospectively analyzed outcomes of LT using livers from donors age 65 years and older and tried to identify those factors that affected graft survival. The results indicated that these factors were hepatitis C as the etiology of liver disease, MELD score higher than 20, donor serum glucose level higher than 200 mg/dL at the time of liver recovery, and skin incision to aortic cross-clamp time longer than 40 min in the donor surgery. In the analysis, the authors observed that the 5-year cumulative graft survival rate of none, one, two, three, and four unfavorable characteristics was 100%, 82%, 81.7%, 39.3%, and 25%, respectively ($P < 0.05$). The authors suggested that the grafts from older donors should not be considerate useless based only on age and that in selected cases, they can result in good graft survival. All these studies are summarized in the Table 1.

Living donor liver transplant

Han *et al*[16] recently demonstrated that living donor LT (LDLT) using elderly donors, defined as those ⩾ 55 years of age, could be related with more serious complications and higher mortality rates. In that retrospective analysis including 604 LDLTs, the

Table 1 Studies that analyze impact of donor age on liver transplant outcomes

Ref.	Type of donor	Cut-off age	No. of patients	Outcomes
Adam *et al*[1]	Deceased donor	< 55 yr *vs* > 65 yr	80347	Higher graft survival with donors younger than 55 yr
Adam *et al*[2]	Deceased donor	Multiple age groups	41522	No differences in one-year survival
Cuervas-Mons *et al*[3]	Deceased donor	55 yr	18568	Lower graft 5-yr survival rate with older donors
Feng *et al*[10]	Deceased donor	60 yr	20023	Higher rate of graft failure with older donors
Reese *et al*[11]	Deceased donor	45 yr	14756	Higher rate of graft failure at 90 d after LT with older donors
Serrano *et al*[12]	Deceased donor	60 yr	149	Lower graft survival rate with older donors
Anderson *et al*[13]	Deceased donor	60 yr	741	No differences were observed
Alamo *et al*[14]	Deceased donor	70 yr	129	No differences were observed in selected recipients (non HCV, low MELD, younger than 60 yr)
Kim *et al*[15]	Deceased donor	65 yr	100	Donor age should not be an absolute contraindication
Han *et al*[16]	Living donor	55 yr	604	Higher mortality rate with older donors
Dayangac *et al*[17]	Living donor	50 yr	150	Higher rate of major complication with older donors
Ikegami *et al*[18]	Living donor	< 30 yr *vs* > 50 yr	34	Better graft function and regeneration rates with donors < 30 yr
Ikegami *et al*[19]	Living donor	50 yr	232	Higher rate of small for size syndrome with older donors
Iwamoto *et al*[20]	Living donor	50 yr	232	Worse survival and high bilirubin levels with older donors
Ono *et al*[21]	Living donor	< 30 yr *vs* > 50 yr	15	Lower regeneration rate a week after LT with older donors
Uchiyama *et al*[22]	Living donor	48 yr	321	Higher rate of small for size syndrome with older donors
Li *et al*[23]	Living donor	70 yr	129	No differences in recipient survival rate at 1, 3 and 5 yr
Wang *et al*[24]	Living donor	50 yr	159	No differences in recipient survival rate at 1, 3 and 5 yr

LT: Liver transplantations; HCV: Hepatitis C virus; MELD: Model for End-stage Liver Disease.

mortality rate was significantly higher in the elderly *vs* the younger donor group. The 5-year survival rate was 44.6% in the elderly group and 80.7% in the younger group, and the median overall survival was significantly shorter in the elderly group (31.2 ± 31.3 mo *vs* 51.4 ± 40.8 mo, $P = 0.014$). Biliary (41.7%) and arterial complications (16.7%) were the more frequent causes of death in the elderly group, which were both significantly higher than in the younger group. This study was limited because of its retrospective analysis that included a small number of patients in the elderly group; nevertheless, the results suggest that donor age directly affects overall survival and complication rate in LDLT.

Another recent study[17] demonstrated a significant association between surgical technique aspects and the rate of major complications when grafts from donors aged ⩾ 50 years are used. In LDLT, enlarging the limits of surgery is associated with more complications in elderly donors. With donors who are ⩾ 50 years old, these authors recommend avoiding right hepatectomy with middle hepatic vein harvesting or resulting in an estimated remnant liver volume less than 35%. Other reports suggest that donor age might have a major effect on recipient outcome in adult LDLT. Ikegami *et al*[18] demonstrated that LT performed with living donors ⩽ 30 years old resulted in better function and regeneration rates within the first month than those performed with donors > 50 years of age. However, the outcome was not affected by the age of the liver graft. In a further study[19], the same authors demonstrated a greater incidence of small-for-size syndrome in recipients from living donors older than 50 years compared to those transplanted with livers from donors ⩽ 50 years old. In addition, Iwamoto *et al*[20] reported significantly higher bilirubin levels and worse survival following transplantations using donors age 50 years or older. Recently, Ono *et al*[21] analyzed hepatic regeneration in living donors and observed that the regeneration rate a week after hepatectomy was significantly higher in donors who were ⩽ 30 years old than in those ⩾ 50 years old; however, the differences disappeared within a month after LT.

These results are consistent with the more recent work of Uchiyama *et al*[22], who retrospectively analyzed 321 consecutive LDLTs performed between 2004 and 2014 and found that donor age was a significant risk factor for small-for-size graft syndrome. In the conclusions, the authors suggest that the use of hepatic grafts from older donors should be avoided if possible to minimize post-transplant complications[22].

On the contrary Li *et al*[23], in a retrospective analysis, found no differences in complication rates and recipient survival at 1, 3, and 5 years. These data suggest that LDLT using older donors had no negative influence on the outcomes of both donors and recipients.

These results are consistent with other recent studies. Wang *et al*[24] analyzed the outcome of 159 LDLTs divided by donor age into older or younger than 50 years and found no significant difference in graft or recipient survival at 1, 3, and 5 years. However, the volume of red blood cells transfused during the surgical procedure was greater in the older donor group (1.900 mL *vs* 1.200 mL, $P = 0.023$). From these results, the authors suggested that LDLT with donors older than 50 years old is safe and that there are not significant adverse effects in terms of graft function and long-term donor and patient survival. All these studies are

Table 2 Variables included in the most relevant survival scores

Model	Variables included	Ref.
DRI	D-age, donor height, DCD, split, race, COD, allocation, CIT	Feng *et al*[10]
ET-DRI	D-age, DCD, Partial/Split, GGT, allocation, rescue allocation	Braat *et al*[25]
SOFT	D-age, COD, donor creatinine, R-age, R-BMI, previous OLT, previous abdominal surgery, R-albumin, dialysis, UNOS status, MELD score, encephalopathy, PVT, ascites, portal bleed, life support, allocation, CIT	Rana *et al*[26]
D-MELD	D-age, MELD score	Halldorson *et al*[27]
BAR	MELD score, CIT, R-age, D-age, previous OLT, life support	Dutkowski *et al*[28]

COD: Cause of death; CIT: Cold ischemia time; DCD: Donation after cardiac death; DRI: Donor risk index; D-age: Donor age; ET-DRI: Eurotrasplant donor risk index; SOFT: Survival outcomes following liver transplantation; R-age: Recipient age; OLT: Orthotopic liver transplant; PVT: Portal vein thrombosis; D-MELD: Donor age Model for End-stage Liver Disease; BAR: Balance of risk.

summarized in the Table 1.

DONOR AGE AS RISK FACTOR IN PROGNOSTIC SCORES

In the past several years, donor quality has been decreasing. Some studies have tried to detect the most important risk factors and to develop several mathematical formulas designed to predict graft outcome. All of them include donor age as a risk factor (Table 2). Feng *et al*[10] performed one of the most relevant studies; this group used the UNOS database to identify eight donor factors predicting graft failure after transplantation (donor age, donor height, donation after cardiac death, split liver donor, black race, vascular accident as cause of death, regional sharing, and cold ischemia time). A donor risk index (DRI) was developed, using these risk factors, to predict the isolated and cumulative effects of these variables on graft survival. Recipients of grafts with a DRI < 1.2 had a graft survival higher than 80% per year *vs* 71.4% in those transplanted with organs with a DRI > 2. In that study, donor age over 60 years was the strongest risk factor for graft failure (relative risk = 1.53 with a donor > 60; 1.65 if > 70). However, this index is not easily applicable in every country. In Europe, Eurotrasplant region database analysis showed that donor age ($P < 0.0001$), donation after cardiac death ($P = 0.001$), split/partial liver ($P < 0.0001$), latest serum GGT gamma-glutamyl transpeptidase ($P = 0.006$), allocation ($P < 0.0001$), and rescue allocation ($P = 0.005$) were significantly associated with an increased risk of graft failure. These six factors were used to construct a "new theoretical Eurotransplant risk index"[25].

Because post-transplantation patient survival depends on both the preoperative medical condition and donor quality, physicians often face the difficult decision of whether to accept high-risk donor liver offers for high-risk patients. Thus, in contrast with DRI, the Survival Outcomes Following Liver Transplantation (SOFT) score includes donor and recipient factors and also ischemia times[26]. The overall result of the score could guide the clinician to either accept or reject the offered allograft, based on the projected risk calculation. Authors proposed that cold ischemia time might be estimated when the offer is performed. Donor age > 70 is the donor variable that has a greater weight in the SOFT score[26]. Halldorson *et al*[27] tried to identify poor donor/recipient matches that could help to direct allocation of organs to recipients in which the survival is greatest, maximizing the benefit of donor livers. They created the D-MELD score, which was calculated as the product of the MELD score and donor age and was demonstrated to be highly predictive of post-LT survival. A D-MELD cut-off of 1600 identified donor/recipient combinations with significantly poorer survival. This score could predict excessive donor/recipient match risk and improve resource use. Another risk score described by Dutkowski *et al*[28] is the balance of risk system, which detects unfavorable combinations of donor and recipient factors. It analyzes six factors including donor age. In summary, donor age is a variable included in all main scores that analyze the risk of death and graft loss after LT and is one of the factors that weighs the most in these models.

LIVER AGE AS RISK FACTOR IN LIVER TRANSPLANT COMPLICATIONS

Donor age also has been described as a risk factor in development of some specific complications such as biliary and aggressive recurrence of HCV. Here we describe the studies that support these data.

Biliary complications

In recent years, numerous studies have shown that donor age may be related to a higher prevalence of biliary strictures. Thorsen *et al*[29] found that LTs performed with donors older than 75 years presented more biliary complications when compared with those patients who received a graft from donors aged 20 to 49 years (29.6% *vs* 13%). However, survival did not differ between groups. Verdonk *et al*[30] found that the incidence of anastomotic strictures (AS) increased from 5.3% before 1995 to 16.7% after 1995, possibly related to an increase in the use of grafts from donors with extended criteria. Similarly, Sundaram *et al*[31] found that biliary AS rate increases after the introduction of MELD for graft allocation (6.4% in the pre-MELD era *vs* 15.4% in the post-MELD era). Transplantation in the post-MELD era was an independent risk factor for biliary AS (OR = 2.30;

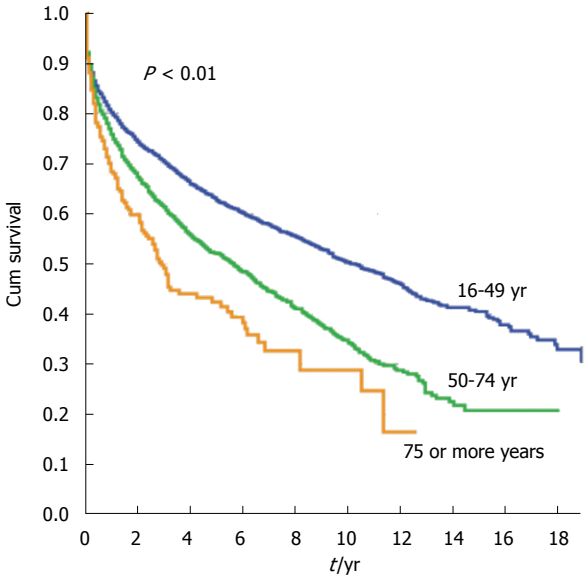

Figure 4 Graft survival in hepatitis C virus-infected patients depending on donor age. Source: Spanish Liver Transplant Registry.

95%CI: 1.60-3.32, $P = 0.001$). Other risk factors were donor age (OR = 1.01; 95%CI: 1.00-1.02, $P = 0.015$), a prior bile leak (OR = 2.24; 95%CI: 1.32-3.76, $P = 0.003$), and a choledochocholedochostomy (OR = 2.22; 95%CI: 1.23-4.06, $P = 0.008$). Nevertheless, in most studies, age was not a risk factor for AS, but it is in non-AS. Lüthold *et al*[32] in a recent study in a pediatric population showed that risk factors for intrahepatic biliary strictures were donor age over 48 years (increase 1.09 fold) and MELD score higher than 30 (increase 1.2 fold). Heidenhain *et al*[33] analyzed nearly 2000 patients retrospectively and found that donor age ($P = 0.028$) and cold ischemia time ($P = 0.002$) were significant risk factors for the development of ischemic-type biliary lesions after liver transplant.

In the study performed by our group and mentioned above, we detected that non-anastomotic biliary strictures (NAS) were four times more frequent in the older donor group. In multivariate analysis (stepwise multiple logistic regression was performed) receiving a graft from a donor 60 years or older (OR = 4.2; 95%CI: 1.24-13.35, $P < 0.01$) and arterial complications (AC) (OR = 67; 95%CI: 11.39-394, $P < 0.0001$) were both independent risk factors associated with NAS. Almost one half of the LT patients with NAS did not have arterial thrombosis. In the logistic regression analysis donor age ⩾ 60 years, emerge as an independent risk factor for intrahepatic non-ischemic strictures (OR = 15.4; 95%CI: 1.42-168.1, $P = 0.024$)[12]. NAS development in these cases could be related to ischemia-reperfusion injury. Despite there were no differences in ischemia time between the two groups it is possible that grafts from older donors were less tolerant to ischemic reperfusion injury. Similar complications have been described with non-beating-heart liver donors; the incidences of both NAS and ischemia-reperfusion injury is higher than with beating-heart donors[34]. Experimental data demonstrated that ischemia-reperfusion injury significantly affects the biliary tree. *In vitro* studies performed on human samples have demonstrated histological and molecular changes in the bile duct that are related to ischemic injury and indicate that biliary tract is the most sensitive structure to this type of injury[35]. Cells from bile duct are more exposed to re-oxygenation damage because they express lower levels of glutathione than hepatocytes[36].

In a recent work, Ghinolfi *et al*[37] demonstrated than LT with liver of donors older than 80 years of age is associated with a higher rate of NAS. Nevertheless the authors suggest that, with appropriate donor/recipient selection, suitable outcomes can be achieved. A higher MELD recipient and donor hemodynamic instability were associated with NAS and poorer graft survival[37].

HCV reinfection

The deleterious effect of donor age on the recurrence of HCV infection has been fully demonstrated. Berenguer *et al*[8] reported that the survival of transplant patients with HCV infection is decreasing, and aging donors is one of the main factors. Donor age is an independent factor associated with the risk of developing cirrhosis and decreased survival. Lake *et al*[38], using data from the American Scientific Registry of Transplant Recipients, analyzed the impact of donor age on the survival of 778 hepatitis B, 3463 hepatitis C, and 7429 non-viral recipients. In HCV-infected recipients, the strongest predictor of graft loss was donor age. Transplantation with organs from donors between ages 41 and 50, 51 and 60, and > 60 years old was associated with a linear increase in the risk of graft loss. Subsequent single or multicenter studies confirmed these findings[39-43]. Analysis of the Spanish Registry for Liver Transplantation presented a lower graft survival in HCV-infected patients when organs were procured from donors older than 50 years[43] (Figure 4). Ghinolfi *et al*[39] analyzed the use of octogenarian donors for LT. In those ⩾ 80 years old, the 5-year graft survival was lower for HCV-positive *vs* HCV-negative recipients (62.4% *vs* 85.6%, $P = 0.034$).

A correlation between accelerated fibrosis and worse outcome in grafts from older donors has been demonstrated[9,44]. Machicao *et al*[9] and Wali *et al*[44] reported that donors age 50 years or more had a median fibrosis progression rate of 2.7 units/year and time to cirrhosis of 2.2 years post-transplant. Donor age was also a strong factor in determining the likelihood of antiviral treatment success[45,46].

The impact on the LT outcomes of new direct-acting antiviral agents (DAA) against HCV has not been well established. These new drugs allow more simple treatment regimens and minimal toxicity, and when used in combination, achieve viral eradication

in most HCV patients who undergo treatment[47,48]. The high cost of DAA still limits treatment on a large scale in most countries. In the next decades, DAA may lead to a significant reduction in patients needing a liver transplant for HCV and improve graft survival rate by decreasing the reinfection rate after LT[49]. In HCV-positive recipients, the impact of donor age on LT outcomes may someday be the same as that if HCV-negative recipients.

LIVER REGENERATION AND AGING

Morphological and structural changes occur in the liver with aging. At the macroscopic level, the liver suffers a reduction in size and a decline in blood flow[50,51]. At the hepatocellular level, changes include a loss of the smooth endoplasmic reticulum, a loss in the number of mitochondria accompanied by an increase in their volume, an increase in the volume of the dense body compartment (secondary lysosomes, residual bodies, lipofuscin), and an increase in hepatocyte polyploidy[52]. Despite morphological changes, the performed clinical studies do not allow for the identification of important age-associated deficits in liver function, and it is generally assumed that the majority of liver functions are relatively well maintained with age[53].

Although age does not seem to significantly affect liver function, the incidence of several liver diseases increases with age whereas the capacity of the liver to manage or overcome liver diseases or external injuries decreases. In fact, the most dramatic and well-documented effect of aging in the liver is the impairment of liver regeneration. Hepatocytes are normally quiescent cells, but in response to liver injury, they can undergo extensive replication to restore the liver. This cellular transition from quiescence to proliferation requires activation of S-phase and mitotic-specific genes. However, fewer hepatocytes in elderly humans enter S-phase in comparison to younger people, and those that do are slower in doing so, compromising the rate of liver regeneration[53].

The loss of liver regenerative capacity is expressed by the decrease in cell cycle and the increase in autophagy and apoptosis[54]. However, despite these phenomena, reported over 50 years ago, the cellular and molecular basis for the loss of an aged liver's regenerative capacity has not been fully elucidated.

Different mechanisms have been suggested as implicated in the loss of this capacity with aging. Reduction in hepatocyte telomere length is one of these suggested mechanisms because it diminishes cell mitosis and apoptosis and thus produces a decline in cell proliferation. Takubo *et al*[55], after studying liver specimens from 94 individuals aged 0-101 years, found significant telomere shortening with age. Similar results were also observed in studies by Aikata *et al*[56] and Aini *et al*[57]. Hepatocytes presenting telomere shortening and karyotypic alterations were found in long-term transplanted human allografts. It appears that telomere shortening in liver cells is more significant in the early years, before the age of 40, when tissue turnover and growth are elevated[55,58]. This timing should be taken into consideration when comparing studies with controversial results because different donor age ranges were used.

Despite the clear connection between telomere shortening and reduction in cell proliferation, this association has not always implied impairment in liver regeneration. Experiments in a telomere restriction fragment-deficient mouse model demonstrated that liver regeneration after partial hepatectomy was not compromised by the loss of telomere integrity[59]. Post-hepatectomy regeneration was accomplished, increasing cell growth and yielding polyploid cells, indicating a switch from a proliferative to a cell growth pathway.

Another factor that suggests involvement of the decline in liver regeneration with aging is the inhibition of regeneration at an epigenetic level. Studies by Timchenko's group[60] indicate that the reduced proliferative response of aged livers is likely to be related to alterations in signal-transduction pathways (at the translational and/or post-translational levels). The decline in the regenerative capacity of old livers seems to be related to epigenetic silencing of E2F-regulated genes as a result of several age-dependent signal-transduction pathways. A decline in growth hormone with age leads to higher cyclin D3 levels that activate cdk4. Activated cdk4 promotes the formation of C/EBPα-Brm and CUGBP1-eIF2 complexes in livers of old mice. CUGBP1-eIF2 complexes up-regulate HDCA1 protein levels that, jointly with C/EBPα-Brm complexes, bind to E2F-dependent promoters, inhibiting expression of E2F-regulated genes and thus liver regeneration. In fact, it has been observed that treatment of old mice with growth hormone corrects liver proliferation[61].

In addition, hepatocellular response to growth factors has been proposed as another mechanism implicated in the reduction of liver regeneration with aging. The hepatocyte proliferative response to epidermal growth factor (EGF) is clearly increased in young rats compared to old animals, suggesting that aging impairs hepatocyte responsiveness to growth factors[53,60]. The problem does not seem to be related to the number of EGF receptors or their binding capacity but rather to a reduction in receptor phosphorylation, a critical step in the EGF-induced hepatocyte proliferation pathway[61].

Apart from the mechanisms mentioned previously, changes in the structure of hepatic sinusoidal endothelium, including a loss of fenestrae and a thickening of the endothelial cells (pseudo-capillarization), have also been associated with a decrease in liver regeneration with aging. Furrer *et al*[62] demonstrated that pseudo-capillarization contributes to age-related decline in regeneration after hepatectomy in mice. Their data demonstrated that treatment with a serotonin receptor agonist in old mice restored liver regeneration capacity

Table 3 Review of cellular and molecular mechanisms suggested to be implicated in the loss of aged liver's regenerative capacity

Mechanism	Ref.
Telomere shortening	Takubo *et al*[55] Aikata *et al*[56] Aini *et al*[57]
Transcriptional and post-transcriptional modifications	Timchenko[60] Wang *et al*[61]
Hepatocelullar response to growth factors	Schmucker[53] Wang *et al*[61]
Pseudo-capillarization	Furrer *et al*[62]
Decline of progenitor cell populations and changes in their niches	Ono *et al*[18] Yousef *et al*[64] Conboy *et al*[65] Wang *et al*[66]

through a vascular endothelial growth factor (VEGF)-dependent pathway. In their findings, the serotonin receptor agonist resulted in increased systemic VEGF availability, up-regulating the number and size of endothelial cell fenestrae, improving hepatic blood flow, and therefore enhancing the hepatic regenerative capacity. In this sense, higher VEGF secretion levels have also been detected in cultures of isolated human hepatocytes from young donors compared to those isolated from older donors[63].

Finally, a decline in the hepatic progenitor cell population has also been suggested as another possible cause of liver regeneration impairment in older donors. Ono *et al*[21] observed that the progenitor cell population (Thy-1$^+$) consistently tended to decline with age in LDLT. On the other hand, Yousef *et al*[64] recently found that the decline in stem cell function with age was largely due to biochemical imbalances in the cell niches, demonstrating that aging imposes an elevation in transforming growth factor β (TGF-β) signaling in the myogenic niche of skeletal muscle and in the neurogenic niche of the hippocampus. When they interfered with TGF-β levels by systemically decreasing TGF-β signaling with a single drug, bringing its levels closer to those detected in young mice, these authors could simultaneously enhance neurogenesis and muscle regeneration in the same old mice, findings further corroborated *via* genetic interference with TGF-β. Conboy *et al*[65] have previously reported similar observations in old mice permanently linked with their vascular systems (heterochronic parabiosis) to young mice. They reported a significant increase in proliferation of the aged hepatocyte progenitors in the old liver and restored expression of the complex c/EBP-alpha to levels seen only in young animals. Additionally, Wang *et al*[66] reported that senescent human hepatocytes can restore their proliferative capacity after xenotransplantation into mice, a finding with a potentially great impact on future studies of liver pathology and liver cell therapy. Hence, a process that once was thought to be terminal - *i.e.*, cell senescence and growth arrest - seems now to be tightly associated with the organ microenvironment rather than with the actual age of the organism. This relationship opens the door to the development of novel pharmacological strategies aimed at rejuvenating old liver grafts immediately after procurement and prior to transplantation.

Thus, understanding the cellular and molecular basis for the reduced proliferative response in old livers is important and could indicate how we can improve liver regeneration and graft survival in older patients. From this perspective, some studies have been designed to find specific markers to predict function and longevity of transplanted organs. Among those senescence markers that have been studied is the abovementioned telomere length; others include the senescence marker protein-30 (SMP-30), which has shown good results in animals that have not correlated with results in humans; CDKN2A/p16INK4a, which is a good predictor of long-term graft function in renal transplantation but has not yet been studied in liver models; the cyclooxygenases 1 and 2 (COX-1 and COX-2); the cell proliferation marker Ki-67; endoplasmic reticulum chaperone levels; and cytochrome p450 mRNA expression[67].

In old animals and in elderly humans liver regeneration is impaired, and it appears to be the rate of liver regeneration, rather than the regenerative capacity, that is diminished in the elderly (Table 3).

These age-related changes could be the factors that determine the higher sensitivity of the graft from older donor to develop irreversible lesions induced by distinct injuries, and results in higher rate of unsuitable response in older donor grafts.

CONCLUSION

The age of donors is increasing significantly in recent years, and liver grafts previously considered suboptimal because they came from elderly donors are nowadays used routinely in all centers. Although the various existing studies so far have contradictory results, age may have a role in the outcome of LT. The use of older donors has been linked to a greater number of biliary complications in both deceased and LDLT, as well as to a poor outcome of HCV recurrence injury. In addition, most LT prognostic scores have donor age as a fundamental variable. The pathophysiological bases of this association are not well established. Liver function does not seem to be influenced by aging, but several changes at the macroscopic and hepatocellular levels have been observed. There are also reported different biological changes in aging that lead to a loss of the liver's proliferative response and regeneration. These alterations may lead to an impairment of the capacity of the liver to manage and overcome liver diseases and to face external injuries.

Donor age is not the only relevant factor in the

outcome of LT, however; surgical factors such as ischemia time or hemodynamic instability during surgery, and recipient factors, such as MELD score, are also essential. Therefore, avoiding these factors as much as possible in liver transplants performed with elderly donors may lead to outcomes similar to those with transplants performed with younger donors.

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REVIEW

Current status of laparoscopic and robotic ventral mesh rectopexy for external and internal rectal prolapse

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Abstract

External and internal rectal prolapse with their affiliated rectocele and enterocele, are associated with debilitating symptoms such as obstructed defecation, pelvic pain and faecal incontinence. Since perineal procedures are associated with a higher recurrence rate, an abdominal approach is commonly preferred. Despite the description of greater than three hundred different procedures, thus far no clear superiority of one surgical technique has been demonstrated. Ventral mesh rectopexy (VMR) is a relatively new and promising technique to correct rectal prolapse. In contrast to the abdominal procedures of past decades, VMR avoids posterolateral rectal mobilisation and thereby minimizes the risk of postoperative constipation. Because of a perceived acceptable recurrence rate, good functional results and low mesh-related morbidity in the short to medium term, VMR has been popularized in the past decade. Laparoscopic or robotic-assisted VMR is now being progressively performed internationally and several articles and guidelines propose the procedure as the treatment of choice for rectal prolapse. In this article, an outline of the current status of laparoscopic and robotic ventral mesh rectopexy for the treatment of internal and external rectal prolapse is presented.

Key words: Laparoscopic ventral mesh rectopexy; Robot; Rectal prolapse; External rectal prolapse; Internal rectal prolapse; Rectocele; Mesh erosion; Obstructed defecation; Faecal incontinence; Biological mesh

Core tip: Globally, there is no uniformity for the treatment of internal and external rectal prolapse. Laparoscopic or robotic-assisted ventral mesh rectopexy is being progressively performed internationally for correcting rectal prolapse. This abdominal approach

avoids posterolateral rectal mobilization and the risks of an anastomosis, corrects the middle compartment, improves anorectal function and shows acceptable recurrence rates. In this article, a synopsis of the current status of laparoscopic and robotic ventral mesh rectopexy for the treatment of internal and external rectal prolapse is presented.

van Iersel JJ, Paulides TJC, Verheijen PM, Lumley JW, Broeders IAMJ, Consten ECJ. Current status of laparoscopic and robotic ventral mesh rectopexy for external and internal rectal prolapse. *World J Gastroenterol* 2016; 22(21): 4977-4987 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i21/4977.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i21.4977

INTRODUCTION

Prolapse of the posterior compartment of the pelvic floor, including rectal prolapse (RP) and its affiliated rectocele and enterocele, is associated with socially debilitating symptoms such as obstructed defecation, pelvic pain and faecal incontinence[1-3]. In the past decades, multiple surgical techniques have been described for RP. There is consensus that perineal procedures are associated with a higher recurrence rate and therefore, an abdominal approach, when possible, is preferred[4,5]. The majority of the abdominal procedures, however, include posterolateral mobilization of the rectum resulting in new-onset or worsening postoperative constipation[6].

In the search to reduce postoperative constipation, ventral mesh rectopexy (VMR) was developed[6]. In this procedure, the rectum is mobilized ventrally and attached to the sacral promontory with a mesh. By avoiding posterolateral rectal mobilization, autonomic nerves are spared and the risk of postoperative constipation is minimised. By lifting the middle compartment of the pelvic floor, correction of other frequently accompanying pelvic prolapses and celes is achieved.

Laparoscopic or robotic-assisted VMR is being progressively performed internationally and several articles and guidelines propose the procedure as the treatment of choice for RP[7-10]. This topic highlight summarises and assesses current evidence on laparoscopic and robotic ventral mesh rectopexy (LVMR/RVMR) for the treatment of internal and external rectal prolapse (IRP/ERP).

SELECTION OF USED LITERATURE

Studies presenting a homogeneous group of patients with rectal prolapse syndromes treated with VMR (laparoscopic or robotic) as described by D'Hoore *et al*[6], avoiding posterolateral mobilization and using a synthetic mesh were selected. Laparoscopic and robotic outcomes had to be displayed separately. Studies describing a heterogeneous group were excluded. Articles using a biological mesh are described separately.

COMPLICATIONS

Laparoscopic

Since the introduction of laparoscopic surgery, complications following rectopexy have reduced significantly[11]. Over the years, many studies have been published investigating surgical complications after LVMR. Most were small case series, but recently a large cohort of 919 patients with a median follow-up of 33.9 mo was published[12]. For this topic highlight, we have included 24 studies showing postoperative complication rates from 0% to 23.5% (Table 1). This extensive variation can be explained by the different ways in scoring morbidity between studies, especially for minor complications. Therefore, we have divided complications in minor and major groups according to the Clavien-Dindo (CD) classification[13]. Major complications, requiring surgical intervention (CD $\geqslant$ 3), are more relevant and in most cases directly ascribed to the VMR. Such complications were demonstrated from 0% to 7.7% of patients, which is acceptable and comparable to other minimal-invasive abdominal pelvic floor procedures[14]. Perioperative mortality is very low and occurred from 0% to 1.1%. Conversion is rare and was described from 0% to 5.9% with one study reporting a rate of ten percent. The majority of the conversions were due to extensive intra-abdominal adhesions.

Robotic

Three studies using synthetic mesh discussed the complication rate following RVMR (Table 1). Robotic surgery showed a non-significant minimal advantage in terms of intra- and postoperative complications compared to LVMR, described in a meta-analysis of these three studies[15]. However, studies were small and follow-up was short. Very recently, a randomised controlled trial (RCT) comparing the two techniques demonstrated a non-significant equality in complication rates[16].

SYNTHETIC MESH-RELATED COMPLICATIONS

Laparoscopic

The use of mesh in pelvic floor surgery has been subject for debate in recent years. Considerable commotion arose in response to the US Food and Drug Administration (FDA) report in 2011 where a high number of mesh-related adverse events associated with transvaginal pelvic organ prolapse repair were described[17]. The systematic review of Abed *et al*[18], showing a mesh erosion rate of 10.3% (110 studies, range 0%-29.7%) within 12 mo after transvaginal pelvic organ prolapse repair, confirmed these concerns.

Table 1 Conversion, intra- and postoperative complications following laparoscopic and robotic ventral mesh rectopexy with synthetic mesh *n* (%)

	n	Median FU (mo)	Intra-operative complications	Conversion	Postoperative complications			
					Total	Minor (CD 1-2)	Major (CD 3-4)	Mortality (CD 5)
Laparoscopic studies								
D'Hoore *et al*[6], 2004	42	61	0	2 (4.8)	2 (4.8)	2 (4.8)	0	0
D'Hoore *et al*[84], 2006	109	-	0	4 (3.7)	8 (7.3)	8 (7.3)	0	0
Slawik *et al*[62], 2008	80	54	-	1 (1.3)	7 (8.8)	7 (8.8)	0	0
van den Esschert *et al*[54], 2008	17	38[1]	0	1 (5.9)	4 (23.5)	3 (17.6)	1 (5.9)	0
Boons *et al*[44], 2010	65	19	-	1 (1.5)	11 (16.9)	6 (9.2)	5 (7.7)	0
Collinson *et al*[51], 2010	75	12	0	1 (1.3)	4 (5.3)	3 (4)	0	0
Wijffels *et al*[85], 2011	80	23	-	1 (1.3)	10 (12.5)	9 (11.3)	1 (1.3)	0
Wong *et al*[36], 2011	40	6	0	4 (10.0)	5 (12.5)	5 (12.5)	0	0
Wong *et al*[52], 2011	84	29	4 (4.8)	3 (3.6)	3 (3.6)	2 (2.4)	1 (1.2)	0
Lauretta *et al*[24], 2012	30	13.9[1]	-	0	2 (7.7)	0	2 (7.7)	0
Faucheron *et al*[86], 2012	175	74/60[2]	0	3 (1.7)	8 (4.6)	5 (2.9)	3 (1.7)	0
Formijne Jonkers *et al*[23], 2013	233	30	0	6 (2.6)	11 (4.7)	7 (3.0)	4 (1.7)	0
Badrek-Amoudi *et al*[25], 2013	48	33	-	0	9 (18.8)	8 (16.7)	1 (2.1)	0
Maggiori *et al*[26], 2013	33	42[1]	0	1 (3.0)	0	0	0	0
Mantoo *et al*[38], 2013	74	16	0	3 (4.1)	15 (20.0)	15 (20.0)	0	0
Mäkelä-Kaikkonen *et al*[37], 2014[3]	20	3	0	0	1 (5.0)	0	1 (5.0)	0
Mackenzie *et al*[32], 2014	953	21	-	8 (1.3)	63 (6.6)	53 (5.6)	8 (0.8)	2 (0.2)
Ogilvie *et al*[30], 2014	29	15.4	1 (3.4)	0	3 (10.3)	2 (6.9)	1 (3.4)	0
Randall *et al*[31], 2014	190	29	1 (0.5)	5 (2.6)	22 (11.6)	11 (5.7)	8 (4.2)	2 (1.1)
Owais *et al*[53], 2014	68	42	0	0	11 (16.2)	10 (14.7)	1 (1.5)	0
Gosselink *et al*[29], 2015	91	12	0	0	5 (5)	4 (4.4)	0	0
Tsunoda *et al*[33], 2015	26	16	0	0	2 (7.7)	2 (7.7)	0	0
Consten/van Iersel *et al*[12], 2015	919	33.9/120[2]	3 (0.3)	20 (2.2)	203 (23.4)	153 (19.3)	50 (4.1)	1 (0.1)
Tsunoda *et al*[34], 2016	31	25	0	0	2 (6.5)	2 (6.5)	0	0
Robotic studies								
Wong *et al*[36], 2011	23	6	0	1 (4.3)	1 (4.3)	0	0	0
Mantoo *et al*[38], 2013	74	16[4]	0	1 (2.3)	5 (11.0)	5 (11.0)	0	0
Mäkelä-Kaikkonen *et al*[37], 2014[3]	20	3	1 (5.0)	0	1 (5.0)	1 (5.0)	0	0

[1]Mean instead of median; [2]Percentages are Kaplan-Meier estimates at 60 and 120 mo of follow-up; [3]The results of Wong, Mantoo and Mäkelä-Kaikkonen *et al* are displayed per technique; [4]Not specified whether mean of median was used. FU: Follow-up; - : Not specified or not applicable.

The contemporary literature present a low incidence of mesh-related morbidity following VMR, but studies discussing this issue are limited. A pooled analysis of 11 observational studies (n = 767) demonstrated a 0.7% rate for mesh-related complications after LVMR in 2012[19]. Recently a multicentre study, including 2203 patients from databases of five hospitals over a 14-year period, described 45 patients (2%) developing mesh erosion (42 synthetic, 3 biologic) after a median of 23 mo[20]. However, underestimation is probable because of the retrospective character and a lack of systematic follow-up of this study. In general, mesh complication rates from 0% to 6.7% with mesh erosion percentages between 0 and 3.7% are described[12,19-34]. Most articles report vaginal mesh erosions, but intrarectal mesh migration following LVMR is not uncommon. The study of Evans *et al*[20] showed approximately half of the mesh erosions were rectal (17/45, 0.8%) and a similar percentage was described in other articles[20,27,31,35]. Recognized risk factors for developing mesh erosion are smoking, steroids, poorly regulated diabetes mellitus, pelvic hematoma, pelvic infection and a history of pelvic irradiation or pelvic surgery[19,35]. The multicentre study also suggested that mesh erosions were more frequently associated with LVMR for IRP (P = 0.02) and polyester mesh (P = 0.00006)[20].

Robotic

Only four studies mentioned examination for synthetic mesh-related complications following RVMR and all reported a rate of zero percent[36-39]. The follow-up, however, was short varying from 3 to 23 mo.

FUNCTIONAL OUTCOME

Laparoscopic - ERP

LVMR, with a limited anterior dissection, was introduced to avoid rectal denervation associated with damage to the parasympathetic fibres of the inferior hypogastric plexus. The RCT of Speakman *et al*[40] showed that preservation, rather than division, of the lateral ligaments is associated with less postoperative constipation. Meta-analyses confirmed this specific finding and demonstrate that VMR seems to be related to less constipation postoperatively as compared with other abdominal techniques (posterior mesh rectopexy, Ripstein, Orr-Loygue)[5,41].

An ERP is a circumferential full-thickness protrusion of the rectum through the anal verge. A recent consensus report, by a panel of international experts,

Table 2 Functional results following laparoscopic and robotic ventral mesh rectopexy with synthetic mesh

Laparoscopic studies	*n*	Median FU (mo)	Improvement OD	*P* value	Improvement FI	*P* value	Median gain CCCS	*P* value	Median gain CCIS	*P* value
Indication ERP										
D'Hoore *et al*[6], 2004	42	61	84.2%. *De novo* 4.8%	-	90.3%	-	-	-	13	< 0.001
Auguste *et al*[21], 2006	54	12	70%. *De novo* 17.6%		72.4%		-	-	5.8[1]	-
Verdaasdonk *et al*[42], 2006[2]	13	7	66%	-	69%	-	-	-	-	-
Cristaldi *et al*[43], 2007	63	18	78%	-	90%. *De novo* 3.2%	-	5	< 0.0001	32 (FISI)	< 0.0001
Boons *et al*[44], 2010	58[3]	19	72%	-	83%. *De novo* 1.5%	-	5	< 0.0001	36 (FISI)	< 0.0001
Formijne Jonkers *et al*[23], 2013[4]	36	30	57.9%	0.01	76.2%	< 0.001	-	-	-	-
Randall *et al*[31], 2014	190	29	-	-	93%	-	-	-	8	< 0.0001
Gosselink *et al*[29], 2015[4]	41	12	-	-	50%	< 0.01	4.8	< 0.01	12 (FISI)	< 0.01
Tsunoda *et al*[33], 2015[4,5]	19	12	52%	-	62%	-	7	< 0.0001	23 (FISI)	< 0.0001
Consten/van Iersel *et al*[12], 2015[4]	242	33.9	63.3%	< 0.0001	73.2%	< 0.0001	-	-	-	-
Tsunoda *et al*[34], 2016	31	12	-	-	-	-	5	0.005	22 (FISI)	< 0.0001
Indication IRP and/or rectocele										
Collinson *et al*[50], 2007	30	3	83%	-	92%	-	9	< 0.0001	25 (FISI)	< 0.0001
Collinson *et al*[51], 2010	75	12	86%	-	85%	-	7	< 0.0001	20 (FISI)	< 0.0001
Wong *et al*[52], 2011	84	29	45%	< 0.001	20%	> 0.05	-	-	-	-
Formijne Jonkers *et al*[23], 2013[4]	197	30	76.9%	< 0.001	65.4%	< 0.001	-	-	-	-
Gosselink *et al*[28], 2013	72	12	-	-	-	-	5	< 0.001	16 (FISI)	< 0.01
Gosselink *et al*[29], 2015[4]	50	12	-	-	48%	< 0.01	3.1	< 0.01	17 (FISI)	< 0.01
Tsunoda *et al*[33], 2015[4,5]	25	12	55%	-	63%	-	6	< 0.0001	22 (FISI)	< 0.0001
Tsunoda *et al*[87], 2015	26	16	-	-	-	-	7	< 0.01	24 (FISI)	< 0.01
Consten/van Iersel *et al*[12], 2015[4]	242	33.9	61%	< 0.0001	73.2%	< 0.0001	-	-	-	-
Indication both ERP and IRP and/or rectocele										
van den Esschert *et al*[54], 2008	1 ERP, 16 IRP	38[1]	-	-	-	-	+2.7[6] (ODS)	0.091	-	-
Lauretta *et al*[24], 2012	2 ERP, 28 IRP	13.9[1]	92.8%	-	85.7%	-	9.1 (ODS)[1][88]	< 0.05	7.1[1]	< 0.05
Badrek-Amoudi *et al*[25], 2013	11 ERP, 37 IRP	33	68%	< 0.0001	-	-	17 (ODS)[7][89]	< 0.0001	4	< 0.0001
Maggiori *et al*[26], 2013	33[8]	42[1]	72%. *De novo* 7%	-	90%	-	-	-	8	0.002
Mackenzie *et al*[32], 2014	149 ERP, 487 IRP	21	56.7%[9]. *De novo* 1.4%	0.119	89.7%[10]. *De novo* 1%	0.040	12 (ODS)[88]	-	8	-
Owais *et al*[53], 2014[11]	18 ERP, 50 IRP	42	82%	-	82%	-	12.5 (ODS)[90]	< 0.001	4	< 0.001
Robotic *vs* Laparoscopic studies - various indications										
De Hoog *et al*[39], 2009[1]	20 ERP *R*	23.4	-	-	-	-	3.2[1]	-	-	-
Mantoo *et al*[38], 2013[12]	23 ERP, 51 IRP *L*	16[13]	-	-	-	-	6 (ODS)[14]	0.004	4[14]	0.604
	12 ERP, 32 IRP *R*						14 (ODS)[14][91]	0.004	4[14]	0.604

[1]Mean instead of median; [2]One patient was excluded from further analysis, therefore *n* = 13 instead of *n* = 14 was used; [3]Functional data were complete in 58 of 65 patients; [4]The results of Formijne Jonkers *et al*, Gosselink *et al*, Tsunoda and Consten and van Iersel *et al* are displayed per indication; [5]Postoperative functional data were fulfilled in 44 of 59 patients; [6]Mean ODS score was 2.7 higher after surgery meaning function deteriorated postoperatively; [7]Pre- and postoperative ODS scores were available for 36 patients; [8]Of the 33 patients (ERP *n* = 20, *n* = 13 IRR) 3 lost to follow-up. For the remainder of patients the surgical indication was not given; [9]Based on 602 patients; [10]Based on 276 patients; [11]Only men included; [12]A modified version of the D'Hoore rectopexy used; [13]Not specified whether mean of median was used; [14]Estimation based on bar chart. OD: Obstructed defecation; FI: Faecal incontinence; ODS: Obstructed defecation syndrome score; L: Laparoscopic; R: Robot; RP: Internal rectal prolapse; ERP: External rectal prolapse; CCCS: Cleveland clinic constipation score; CCIS: Cleveland clinic incontinence score.

considers ERP a definitive indication for VMR[9]. LVMR showed improvement of obstructed defecation from 52% to 84.2% ($P < 0.01$-$P < 0.0001$)[6,12,21,23,33,42-44] with a median gain of the Cleveland Clinic Constipation Score (CCCS)[45] between 4.8 and 7 points ($P < 0.01$-$P < 0.0001$, Table 2)[29,33,34,43,44]. Obstructed defecation *de novo* was noted in 4.8% to 17.6% of patients[6,21]. Improvement of faecal incontinence was described in 50% to 93% of patients ($P < 0.01$-$P < 0.0001$)[6,12,21,23,29,31,33,42-44]. There was a median gain of the Cleveland Clinic Incontinence

Score (CCIS)[46] of 8 to 13 ($P < 0.001$ - $P < 0.0001$)[6,31] and one study reports a mean CCIS gain of 5.8 points[21]. The median Faecal Incontinence Severity Index (FISI)[47] benefit varied from 12 to 36 points ($P < 0.01$ - $P < 0.0001$)[29,33,34,43,44]. Two studies demonstrated new-onset faecal incontinence with an incidence of 1.5% and 3.2% in patients[43,44].

Laparoscopic - ERP and/or IRP and/or rectocele

An IRP is a telescopic infolding of the rectal wall during defecation. IRP is most commonly classified by the Oxford rectal prolapse grade differentiating between an intrarectal (grade 1 and 2) and intra-anal (grade 3 and 4) intussusception[48]. An Oxford grade 3 or 4 IRP, in combination with significant functional complaints failing to conservative therapy, is considered an indication for VMR[9,10]. The expert panel stated that VMR could also be performed for a complex rectocele of more than 3-4 cm[9]. However, a rectocele frequently exists with an IRP (80%) and, therefore, an isolated rectocele is rare (10%)[49]. LVMR for IRP and/or rectocele showed improvement of obstructed defecation from 55% to 86% ($P < 0.001$ - $P < 0.0001$)[12,23,33,50-52] with a median CCCS decrease between 3.1 and 9 points ($P < 0.01$ - $P < 0.0001$, Table 2)[28,29,33,34,50,51]. Improvement of faecal incontinence with 20% to 92% of patients ($P > 0.05$ - $P < 0.0001$)[12,23,29,33,50-52] and a median gain of FISI of 16 to 25 points ($P < 0.01$ - $P < 0.0001$) was observed in multiple cohorts[28,29,33,34,50,51]. None of the studies performing LVMR for IRP and/or rectocele described new-onset functional complaints.

Studies including both ERP and IRP and/or rectocele as indication for surgery showed 56.7% to 92.8% ($P = 0.119$ - $P < 0.0001$)[24-26,32,53] improvement for obstructed defecation complaints with a median advantage of 9.1 to 17 points in obstructed defecation syndrome (ODS) score ($P < 0.05$ - $P < 0.0001$, Table 2)[24-26,32,53]. One report described a non-significant deterioration in ODS score postoperatively[54]. A decrease in faecal incontinence complaints is reported from 82% to 90% of patients [24,26,32,53] with a median CCIS gain of 4 to 8 points ($P < 0.05$ - $P < 0.0001$)[24-26,32,53]. Literature demonstrated new-onset complaints of obstructed defecation between 1.4% and 7% of patients, with one report showing a *de novo* faecal incontinence rate of one percent[26,32].

Robotic

To date, only two studies using synthetic mesh discuss functional outcomes following RVMR (Table 2)[38,39]. The laparoscopic cohort of de Hoog *et al*[39] included various mobilizations and was excluded. The RVMR series of this study showed a median CCCS gain of 3.2 points, which was lower than other studies performing LVMR for ERP (Table 2). Mantoo *et al*[38], performing LVMR and RVMR for various indications, noted a significantly greater improvement for obstructed defecation after RVMR. Median gain of CCIS was non-significantly equivalent between the two techniques. Both improvement of obstructed defecation and faecal incontinence was in line with the literature on LVMR for various indications (Table 2). Functional outcome was not described in the recent RCT of Mäkelä-Kaikkonen *et al*[16]. However, this RCT did show a non-significant difference in postoperative residual rectoceles on MRI in favour of the robot compared with laparoscopy (8% *vs* 33%, $P = 0.26$). This may result in a better functional outcome for patients suffering obstructed defecation, but these outcome measures need to be evaluated at a longer follow-up.

RECURRENCE

Laparoscopic - ERP

With the introduction of minimally invasive surgery, recurrence rates with rectal prolapse surgery remained low and equivalent to those of open surgery[55,56]. In the nineties, three small trials suggested that preservation of the lateral ligaments might result in a higher recurrence rate[11]. Tou *et al*[11] speculated this was due to the limited mobilization of the rectum. Nonetheless, to date, numerous non-randomised observational studies, with increasing follow-up, quote acceptable recurrence rates following VMR. From 2004 until presently, recurrence percentages following LVMR for ERP range between 1.5% to 9.7%, with one small cohort ($n = 13$) reporting a rate of 15.4% (Table 3). Several reviews demonstrate comparable recurrence rates with various rectal mobilisations and abdominal techniques[5,22,41]. In addition, a multicentre, pooled analysis of 643 patients from 15 centres undergoing abdominal surgery for ERP, showed that the method of rectopexy did not influence the recurrence rate[57].

Variation in recurrence usually reflects differences of follow-up between studies. Articles reporting on LVMR for ERP described a time interval to presentation of recurrence between 10 and 91 mo after surgery. Most recurrences developed within the first 36 mo, but not all studies reported this time interval (Table 3). Little is known about risk factors for developing a recurrence following VMR. Mackenzie *et al*[32] found the only predictor of recurrence was the use of polyester mesh which generated a twofold increase in recurrence rate, with an odds ratio of 1.96 ($P = 0.017$), as compared with the most commonly used polypropylene graft.

Laparoscopic - ERP and/or IRP and/or rectocele

Three studies, performing LVMR for IRP and/or rectocele, quoted recurrence rates between 5.3 and 7.1 percent. Literature, including all rectal prolapse syndromes, reported recurrence percentages between 2.6% to 14.3%. The time interval between LVMR for various indications and recurrence varied from 10 to 139 mo (Table 3).

Table 3 Recurrence rates following laparoscopic and robotic ventral mesh rectopexy with synthetic mesh *n* (%)

Laparoscopic studies	*n*	FU (median)	Recurrence	Type of recurrence	Presentation of recurrence (mo)
Indication ERP					
D'Hoore *et al*[6], 2004	42	61	2 (4.8)	2 ERP	54, 91
Verdaasdonk *et al*[42], 2006[1]	13	7	2 (15.4)	2 ERP	-
Auguste *et al*[21], 2006	54	12	4 (7.4)	3 ERP, 1 IRP	26 (7-54)[2]
D'Hoore *et al*[84], 2006	109	-	5 (4.6)	4 ERP, 1 enterocele	-
Cristaldi *et al*[42], 2007	63	18	1 (1.7)	ERP	-
Boons *et al*[44], 2010	65	19	1 (1.5)	ERP	12
Wijffels *et al*[85], 2011	80	23	2 (2.5%)	2 ERP	6, 16
Faucheron *et al*[86], 2012	175	74/60[3]	2 (3)[3]	2 ERP	6, 24
Randall *et al*[31], 2014[5]	190	29	9 (4.7)	1 ERP, 8 IRP	25, 30, 31, 60[6]
Gosselink *et al*[29], 2015[3]	41	12	1 (2.3)	ERP	12
Tsunoda *et al*[34], 2016	31	25	3 (9.7)	3 IRP	10, 17, 31
Indication IRP and/or rectocele					
Collinson *et al*[51], 2010	75	12	4 (5.3)	4 IRP	-
Wong *et al*[52], 2011	84	29	6 (7.1)	6 rectocele	-
Gosselink *et al*[29], 2015[4]	50	12	3 (5.8)	3 IRP	-
Indication both ERP and IRP and/or rectocele					
Lauretta *et al*[24], 2012	2 ERP, 28 IRP	13.9[7]	1 (3.3)	1 IRP	19
Formijne Jonkers *et al*[23], 2013	36 ERP, 197 IRP	30	6 (2.6)	-	-
Badrek-Amoudi *et al*[25], 2013	11 ERP, 37 IRP	33	4 (8.3)	4 IRP	22 (median)
Maggiori *et al*[26], 2013	33[8]	42[7]	2 (6.7)	2 rectocele	11, 14
Mackenzie *et al*[32], 2014	149 ERP, 487 IRP	21	60 (9.4)	-	-
Owais *et al*[53], 2014[9]	18 ERP, 60 IRP	42	2 (2.9)	2 IRP	-
Consten/van Iersel *et al*[12], 2015	242 ERP, 677 IRP	33.9/120[3]	68 (14.3)[3]	15 ERP, 53 IRP	24.1 (1-139.4)[2]
Tsunoda *et al*[33], 2015	19 ERP, 25 IRP	26	2 (3.4)	2 IRP	10, 15
Robotic *vs* Laparoscopic - various indications					
De Hoog *et al*[39], 2009	20 ERP robot	23.4	4 (20)	-	-
Wong *et al*[92], 2011	23 IRP lap	12	1 (4.3)	Rectocele	3
	15 IRP robot		1 (6.7)	Rectocele	7
Wong *et al*[36], 2011	40 IRP lap	6	0 (0)	-	-
	23 IRP robot		0 (0)	-	-
Mantoo *et al*[38], 2013[10]	23 ERP, 51 IRP lap	16[11]	6 (8)	-	-
	12 ERP, 32 IRP robot		3 (7)	-	-
Mäkelä-Kaikkonen *et al*[37], 2014	14 ERP, 6 IRP lap	3	1 (5)	-	-
	13 ERP, 7 IRP robot		0 (0)	-	-

[1]One patient was excluded from further analysis, therefore *n* = 13 instead of *n* = 14 is used; [2]Mean (range); [3]Recurrence percentage is KM estimate at 60 and 120 mo of follow-up; [4]The results of Gosselink *et al* are displayed per indication; [5]Study group included the first 44 cases from Slawik *et al*[62]; [6]Only 4 time intervals are described; [7]Mean instead of median; [8]Of the 33 patients (ERP *n* = 20, *n* = 13 IRR) 3 lost to follow-up. For the remainder of patients the surgical indication was not given; [9]Only men included; [10]A modified version of the D'Hoore rectopexy used; [11]Not specified whether mean of median was used. Lap: Laparoscopic; IRP: Internal rectal prolapse; ERP: External rectal prolapse.

Robotic

The contemporary literature comparing LVMR with RVMR show similar recurrence rates between the two techniques (Table 3). Recurrence percentages vary from 0 to 7 for the robotic and 0 to 8 for the laparoscopic inclusions and were comparable to observational LVMR studies. One additional study from the de Hoog *et al*[39] noted a recurrence rate of 20% for the robotic, and 26.7% for the laparoscopic cohort. The laparoscopic series also included Well's procedures and therefore these results were excluded for analysis (Table 3).

MULTI-COMPARTMENT PROLAPSE

Pelvic floor dysfunction is regularly characterised by multi-visceral pelvic organ prolapse[58]. With an ageing population, the prevalence of uni- and multi-visceral pelvic organ prolapse will increase[59-61]. A growing number of articles discuss a multidisciplinary approach for multi-compartment prolapse, but only two studies avoid posterolateral rectal mobilization[62,63]. The first report, describing an open recto-vagino-vesicopexy, presented an improvement with constipation in 77% (P = 0.001), faecal incontinence in 69% (P = 0.005) and urinary incontinence in 50% (P = 0.18) of all patients respectively after 12 mo[63]. Two (8%) patients developed new-onset urinary incontinence. Slawik *et al*[62], performing a laparoscopic sacro-colpo-rectopexy, described an improvement in 91% of patients with faecal incontinence and a reduction in median CCIS of 10 points after six months. Obstructed defecation resolved in 80% of patients, but 7% of these underwent an additional bowel resection. New-onset obstructed defecation occurred in 3.8%, and urinary incontinence in 2.5% of patients respectively. No patient developed a recurrence after a median follow-up of 54 mo. Thus far, no robotic studies describing a

multi-compartmental approach with a limited anterior rectal dissection are published.

BIOLOGICAL MESH

Concerns over synthetic mesh-related complications such as erosion, dyspareunia, fistulation and stricturing have led to the introduction of a more expensive biological equivalent. The biological mesh is characterised by degradation of the graft and regeneration of host tissue[64]. In theory, this degradation could decrease the chance of erosion and chronic infection. Conversely, the partial resolution of the material may lead to a higher recurrence rate. In 2013, a systematic review by Smart *et al*[19] was published comparing 11 studies (767 patients) receiving synthetic mesh with two studies (99 patients) using a biologic graft. An erosion rate of less than one percent, with no difference identified between synthetic and biological mesh (0.7% *vs* 0%, P = 1.0%) was described. There was no significant difference in other mesh-related complications or short-term recurrence (3.7% *vs* 4.0%, P = 0.78). The multicentre study of Evans *et al*[20] and two recent biological mesh studies[65,66] (4 and 20 mo follow-up) showed similar rates of mesh erosion. However, Franceschilli *et al*[66] reported a much higher percentage prolapse recurrence rate of 14% after a mean follow-up of 20 mo. Improvement with obstructed defecation was described from 82% to 92% with a mean gain of CCCS between 9 and 13 points (P = 0.02 - P < 0.0001)[65-68]. Reduction in faecal incontinence complaints occurred in 73% and 85% of patients with a mean gain in FISI score between four and 6 points (P = 0.01-P = 0.001)[65-68]. One report demonstrated a median gain in CCIS of approximately 10 points (P = 0.0002)[65-68]. Wahed *et al*[67] was the only study describing new-onset complaints (4.6% with constipation and 3.1% with faecal incontinence). Only one study comparing and matching biological and synthetic mesh for LVMR (29 *vs* 29) excists, demonstrating no significant difference in mesh-related complications, recurrence or functional outcome after a median follow-up of 15.4 mo[30]. Mehmood *et al*[69], comparing 34 LVMR with 17 RVMR patients using biological mesh, demonstrated a minor significant advantage in median CCIS gain for LVMR (10 *vs* 9.5, P = 0.02). Conversely, a non-significant benefit in favour of the robot was seen in a reduction of the FISI (32 *vs* 35, P = 0.3). Both the functional outcomes of the robotic and the laparoscopic cohort compared favourably to other studies describing LVMR for ERP (Table 2). No recurrences or mesh-related complications were seen in either cohort after 12 mo. There is a lack of high-level comparative evidence with long-term follow-up for biological mesh, which demonstrates any significant difference in graft-related morbidity and recurrence rates. When more data becomes available, the choice of the mesh may be influenced by cost or possible comorbidity. In a recent publication a panel of experts suggested that biological grafts may be a better option in the following circumstances: young patients, women of reproductive age, diabetics, smokers, patients with a history of previous pelvic radiation or sepsis, inflammatory bowel disease, and in cases of intraoperative breach of the rectum or vagina[9].

DISCUSSION

LVMR has become popularised in the past decade and is the preferred technique for treating rectal prolapse syndromes by many surgeons, especially in Europe. The procedure is becoming increasingly applied with robotic assistance. The robot enhances visualisation and manoeuvrability to improve complicated procedures in the deep pelvis, such as dissection and intracorporeal suturing[70]. Robotic surgery has proven to be more expensive in the short-term, but may lead to an overall reduction of costs due to enhanced ergonomics for the surgeon[11,71,72]. However, a long-term cost-effectiveness analysis of LVMR *vs* RVMR has not been performed.

The current evidence shows that LVMR and RVMR are safe procedures in terms of intraoperative, post-operative and mesh-related complications. Both LVMR and RVMR generate an acceptable recurrence rate and satisfactory improvement of functional outcome, with only one small laparoscopic cohort reporting an overall non-significant deterioration with obstructed defecation after surgery[54]. There may be a trend towards a better outcome for obstructed defecation following RVMR as compared with LVMR, but the level of evidence is low[16,38]. LVMR and RVMR show similar good results for improvement of faecal incontinence. Based on the currently available data, no superiority for either technique can be determined. As compared with other observational studies describing an abdominal approach to treat rectal prolapse syndromes, VMR shows similar recurrence rates and less constipation postoperatively[5,22,41]. Circumspection is required interpreting these results, however. Heterogeneity between the articles in patient selection and outcomes measured makes it difficult to draw conclusions from the current literature. In addition, follow-up has been relatively short and lacks a systematic approach for the majority of studies, especially the robotic series. Since pelvic floor dysfunction increases with age, long-term follow-up is required to assess functional outcome and recurrence. The true mesh erosion rate can only be obtained with adequately powered, long term studies incorporating a vaginal and anorectal examination for every patient. Thus far, only level 3 evidence exists with a paucity of RCT's and case controlled trials. There are no results of comparative studies including VMR available. In a recent critical appraisal, Lundby and Laurberg expressed their concerns about the rapid implementation of LVMR for obstructed defecation syndrome based on the contemporary evidence[73].

High-level comparative evidence is necessary to overcome these doubts and to determine the value of VMR in the definitive treatment of rectal prolapse syndromes.

FUTURE RESEARCH

This review focusses solely on VMR, but more than three hundred different procedures to treat rectal prolapse syndromes have been described. Thus far, no technique has been shown to be superior. This was confirmed by an international survey in 2012, showing no uniformity of surgical procedure[74]. The survey demonstrated, inter alia, that more than 20% of the surgeons preferred stapled transanal resection of the rectum (STARR) for the treatment of IRP. Festen *et al*[75] suggested an IRP associated with fecal incontinence should be treated with LVR and an IRP in combination with obstructed defecation with STARR or LVR. At present, one Italian trial is comparing LVMR with STARR for obstructed defecation syndrome[76]. In addition, there are eight ongoing surgical trials, of which five are mentioned by the cochrane study of Tou *et al*[11]; two comparing LVMR with Delorme's procedure for ERP[77,78], one investigating the outcomes of LVMR versus laparoscopic posterior rectopexy without mesh[79], one comparing laparoscopic resection rectopexy (RR) with laparoscopic fixation rectopexy[80], one assessing the difference between standard mesh rectopexy with ventral rectopexy[81], one studying the efficacy of LVMR for the treatment of chronic constipation[82] and two examining LVMR versus RVMR[16,83]. The trial by Mäkelä-Kaikkonen *et al*[16] has presented its short-term results, but long-term outcomes are awaited. The survey also shows VMR and RR are the two most common abdominal procedures for RP[74]. RR was developed to reverse the symptoms of rectal denervation inertia which is associated with traditional posterolateral rectal mobilization, but introduces the risks of a pelvic anastomosis. Three trials, comparing (predominantly open) abdominal rectopexy with and without sigmoid resection, described that RR was associated with less postoperative constipation but with a higher complication rate ($P > 0.05$)[11]. There is a need for a well-designed and adequately powered RCT comparing these two techniques laparoscopically or robotic-assisted. Lastly, high-quality evidence for the choice of a specific mesh type, either synthetic or biological, is required. The authors do acknowledge the slow recruitment and logistical difficulties of performing such trials, however.

CONCLUSION

Ventral mesh rectopexy (laparoscopic and robotic) appears a safe and effective procedure to correct different rectal prolapse syndromes with a low morbidity rate, acceptable long-term recurrence rates and a good functional outcome.

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ORIGINAL ARTICLE

Basic Study

Multiorgan chronic inflammatory hepatobiliary pancreatic murine model deficient in tumor necrosis factor receptors 1 and 2

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Abstract

AIM: To provoke persistent/chronic multiorgan inflammatory response and to contribute to stones formation followed by fibrosis in hepatobiliary and pancreatic tissues.

METHODS: Tumor necrosis factor receptors 1 and 2 (TNFR1/R2) deficient mice reared in-house were given dibutyltin dichloride (DBTC) twice within 10 d by oral gavage delivery. Sham control animals received vehicle treatment and naïve animals remained untreated throughout the study. Animals were monitored daily for symptoms of pain and discomfort. The abdominal and hindpaw hypersensitivity were assessed with von Frey microfilaments. Exploratory behaviors were recorded at the baseline, after initiation of treatment, and before study termination. Histopathological changes were examined postmortem in tissues. Collagen accumulation and fibrosis were confirmed with Sirius Red staining.

RESULTS: Animals lost weight after oral administration of DBTC and developed persistent inflammatory abdominal and hindpaw hypersensitivity compared to sham-treated controls ($P < 0.0001$). These pain related secondary mechanical hypersensitivity responses increased more than 2-fold in DBTC-treated animals. The drastically diminished rearing and grooming rates persisted after DBTC administration throughout the study. Gross as well as micropathology at one month confirmed that animals treated with DBTC developed chronic hepatobiliary injuries evidenced with activation of stellate cells, multifocal necrosis, fatty degeneration

of hepatocytes, periportal infiltration of inflammatory cells, and prominent biliary ductal dilation. The severity of hepatitis was scored 3.7 ± 0.2 (severe) in DBTC-treated animals *vs* score 0 (normal) in sham-treated animals. Fibrotic thickening was extensive around portal ducts, in hepatic parenchyma as well as in lobular pancreatic structures and confirmed with Sirius Red histopathology. In addition, pancreatic microarchitecture was presented with distortion of islets, and parenchyma, infiltration of inflammatory cells, degeneration, vacuolization, and necrosis of acinar cells and distention of pancreatic ducts. Extent of pancreatic damage and pancreatitis were scored 3.6 ± 0.4 (severe) for DBTC-treated in contrast to score 0 (normal) in sham-treated animals. The gall bladder became expanded with ductal distention, and occasional bile stones were detected along with microscopic hepatic lesions. DBTC-treated animals developed splenic hypertrophy with increased weight and length ($P < 0.01$) along with thymic atrophy ($P < 0.001$). Finally, colitic lesions and colitis were prominent in DBTC-treated animals and scored 3.4 ± 0.3 (moderately severe) *vs* 0 (normal) for the sham-treated animals.

CONCLUSION: This is the first report of chronic inflammatory multiorgan hepatobiliary pancreatitis, along with fibrosis and calculi formation induced reliably utilizing oral DBTC administration in TNFR1/R2 deficient mice.

Key words: Inflammatory pain; Multiorgan; Hepatitis; Pancreatitis; Calculi formation; Gall bladder; Hepatobiliary inflammation

Core tip: Currently there is no reliable model for chronic multiorgan inflammatory and fibrosis. Tumor necrosis factor (TNF)α initiates inflammation through TNFR1/R2. TNFR1/R2 deficient mice administered orally with dibutyltin dichloride (DBTC) developed significant persistent inflammatory and pain related secondary mechanical hypersensitivity. DBTC-animals showed severe chronic hepatobiliary injuries and prominent biliary ductal dilation. Extensive fibrotic thickening was evidenced around portal ducts, in hepatic and pancreatic structures. DBTC-animals had severe pancreatic damage and pancreatitis, hepatic lesions with expansion of gall bladder, bile stones and severe colitis. This is the first report of chronic inflammatory multiorgan hepatobiliary pancreatitis, fibrosis and calculi formation in TNFR1/R2 deficient mice.

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INTRODUCTION

Fibrogenesis is a required process in wound healing, but persistent inflammatory and fibrotic reaction can lead to devastating symptoms and eventually organ failure[1,2]. Multiorgan fibrosis is typically the end product of various unresolved or repetitive tissue injuries from chronic inflammation, infection, radiation exposure, and abnormal repair outcome. Loss of function contributes to progression of morbidity and mortality. Multiorgan fibrosis is a common complication in cystic fibrosis[3], systemic sclerosis[4] and primary sclerosing cholangitis[5]. Chronic pancreatitis, initiated by idiopathic or recurrent inflammation, is manifested with irreversible destruction of exocrine parenchyma and pancreatic fibrosis. It is a potentially fatal progressive disease leading to diabetes mellitus and pancreatic cancer. Pancreatitis is associated with spontaneous visceral pain as a chief symptom in patients. Neural innervation of the pancreas is pivotal in the instigation and continuation of inflammation and pain response. Cellular destruction leads to activation of pancreatic sensory neurons causing release of neurotransmitters in the spinal cord and neurogenic signaling then back to the pancreas provoking plasma extravasation and neutrophil infiltration[6].

Multifactoral gallstones are one of the most prevalent gastrointestinal complications with serious outcomes such as gallstone pancreatitis and cancer. Gallstone disease is a chronic recurrent hepatobiliary complication which is characterized by formation of gallstones in the hepatic and bile duct, or gallbladder. It is manifested by impaired metabolism of cholesterol, bilirubin and bile acids[7].

Tumor necrosis factor α (TNFα) a proinflammatory cytokine, up-regulates various cytokines/chemokines to initiate acute and chronic stages of inflammation. The biological action of TNFα is chiefly through two gene family receptors, TNFR1 and TNFR2. TNFα is released mainly by activated macrophages, in addition to astroglia, microglia, CD4+ lymphocytes, Natural killer cells (NK), and neurons[8-10]. The complete-length membrane-crossing TNFα (mTNFα) is sliced by the inducible TNF converting enzyme (TACE) to release soluble TNFα (sTNFα) and diffusible peptide[11]. TNFα release is associated with inflammatory response and pain related sensation in patients with pancreatitis, hepatitis and inflammatory bowel disease (IBD), as well as neuropathy[12]. TNFα contributes to development of neuropathic pain[13]. Soluble TNFR1 and R2 neutralize circulating TNFα to alleviate pain related responses to mechanical allodynia, thermal hyperalgesia or peripheral nerve injuries[14-16]. TNFα plays an important function in the pathogenesis of acute pancreatitis. Recent investigations have demonstrated that TNFα inhibition drastically ameliorates the duration of experimental acute pancreatitis[17]. TNFα receptor 1 (TNFR1) gene deletion and etanercept application likewise ameliorated the duration of acute pancreatitis

in animal models, suggesting potential of etanercept and anti-TNFα monoclonal antibodies as therapy in clinical pancreatitis[17]. Although, current clinical treatments with these biological agents may diminish inflammation and pain by reducing TNFα and other cytokines, the inflammatory response and pain is likely to re-emerge in most patients with autoimmune disease including arthritis and IBD[10]. In addition, anti-TNFα monoclonal antibodies therapy has potential side effects such as provoking infections with JC virus, fungi and tuberculosis. Currently there is no cure or reliable mouse model for chronic pancreatitis.

Previously we have demonstrated that the baseline mechanical and thermal response to noxious stimulation is similar in TNFR1/R2 deficient mice *vs* wildtype background mice. However, TNFR1/R2 deficient mice develop more severe responses when similarly treated with various insults[10,16]. Animal models of acute and chronic pancreatitis have been utilized to examine mechanisms of pathogenesis, and to test possible therapeutic interventions. One of the most commonly used pancreatitis models is created by serial intraperitoneal administration of concentrations of caerulein, an ortholog of cholecystokinin[17]. Other chemically induced models have utilized di-n-butyltin dichloride (DBTC). DBTC is a polyvinyl carbonate (PVC) plastic stabilizer/catalyzer additive, insecticide and biocide in agriculture, and antifouling agent in the paint and fabric industry that often contaminates food and water[18]. Tail vein slow injection of DBTC induces relatively unpredictable pancreatitis flares in rats[6]. However, DBTC injection is tedious and minor leakage results in tail necrosis, gangrene and animal loss. We hypothesized that oral administration of DBTC would provoke persistent and chronic pancreatitis in animals deficient in TNF-receptors. Similarly, TNFR1/R2 may accelerate inflammatory response in multiorgans and contribute to stones formation and fibrosis in hepatobiliary and pancreatic tissues. Here we report a chronic persistent DBTC-induced inflammatory model by oral gavage in TNFR1/R2 deficient mice persisting at least one month allowing more clinically relevant studies in this model. Pain related behaviors accompanying this model are characterized.

MATERIALS AND METHODS

Animals

All animal procedures were approved by the University of Kentucky Institution Animal Care and Use Committee (IACUC). Mice were monitored daily for continued weight gain/loss and general health. Health status and procedures were documented daily on the UK IACUC Standard Operating Procedure (SOP-102) Post-Operative Evaluation form. Experiments were performed using dually deficient TNFR1/R2 mice (Jackson Laboratory) on a B6129SF2/J background inbred at the University of Kentucky animal facilities and provided by Dr. Westlund. Mice were housed in individual cages with a 10 h/14 h dark/light reversed cycle to accommodate behavioral test during their active dark period. Mice were allowed free access to food and water ad libitum, except 2 h before and during behavioral testing.

Induction of persistent chronic pancreatitis

Chronic persistent pancreatitis was induced in mice utilizing DBTC (Dibutyltin dichloride, Sigma-Aldrich, St Louis, MO). DBTC (10 mg/kg) was dissolved in 95% ethanol (two parts) and then mixed with glycerol (three parts) and given orally. Mice received DBTC by oral gavage (200 μL volume). Intragastric gavage administration was performed by Dr. Oz, an expert veterinarian scientist, in conscious animals, using appropriate bended gavage needles (22 gauge, 1 inch length, 1.25 mm ball diameter). Sham control mice were given the vehicle (95% ethanol + glycerol, 2:3) alone and Naïve control animals remained untreated. Animals were monitored until fully active. In order to induce chronic inflammation, mice received a 2nd treatment by oral gavage within 10 d. Following induction of pancreatitis the animals were monitored daily for activity, appearance, and signs of abdominal discomfort. They were weighed regularly and tested for hypersensitivity on the hindpaw plantar foot pad and the shaved abdominal surface. After completion of the final behavioral testing, one month after induction, the animals were euthanized with isoflurane overexposure, the thorax opened, blood samples collected by cardiac puncture, and tissue samples collected for histological evaluation.

Assessment of secondary mechanical allodynia by testing hindpaw withdrawal threshold

Pain-related behavior was assessed throughout the study by the determining secondary mechanical threshold to assess hyperalgesia/allodynia. The von Frey test is a standard comparison used in the field of pain research. Day 0, baseline testing to determine footpad nociceptive responses was performed testing hindpaw withdrawal latency to mechanical stimuli with von Frey fibers. Reflex testing for secondary mechanical hyperalgesia/allodynia with von Frey fibers was developed by Max von Frey, who in 1896 identified "pain spots" on human skin. Mechanical nociceptive thresholds were analyzed as described previously[10,19]. Paw withdrawal response latencies were assessed weekly throughout the study. Mice were placed into clear cylindrical plastic enclosures (7 cm × 4 cm × 4 cm) on a smooth metal meshed (3 mm × 3 mm) platform (36 cm × 29 cm × 21.5 cm). Mechanical withdrawal threshold testing was done on the plantar surface of both hindpaws using a set of 8 von Frey monofilaments [(4.74) 6.0 g; (4.31) 2.0 g; (4.08) 1.0 g; (3.61) 0.4 g; (3.22) 0.16 g; (2.83) 0.07 g; (2.36) 0.02 g; (1.65) 0.008 g]. The von Frey

filaments were applied perpendicularly to the plantar surface with sufficient force to bend the monofilament slightly and held for about 5 s, and 5 to 10 times with 15 s intervals. A positive response was defined as an abrupt withdrawal (flick response) of the foot during stimulation or immediately after the removal of stimulus. Whenever there was a negative or positive response, the next stronger or weaker filament was applied, respectively. Testing proceeded in this manner until four fibers had been applied after the first one caused a withdrawal response, allowing the estimation of the mechanical withdrawal threshold.

Pain-related behavioral evaluations for abdomen: Prior to induction of inflammation with DBTC administration, baseline testing of abdominal nociceptive responses to mechanical stimuli was performed with von Frey fibers applied to the upper left abdominal quadrant skin of mice as previously described[10,19]. Mechanical hypersensitivity in the abdominal area was quantified by measuring the number of withdrawal events (either abdominal withdraw from the von Frey filament or consequent licking of the abdominal area, or whole body withdrawal) in response to normally innocuous or sub-threshold mechanical stimuli. Testing continued weekly throughout the study.

Evaluation of the pain-related posture: The abnormal posture of each animal with an affected hindlimb was given a single score using a subjective pain-related behavioral scale (spontaneous pain rating score 0-5) *i.e.* 0- normal; 1- curling of the toes, 2- aversion of the paw; 3- partial weight bearing; 4- non-weight bearing and guarding; and 5- avoidance of any contact with the hindlimb.

Pain-related gait disturbance: Gait disturbances (curling toes, limping, guarding, rearing and grooming were tallied by an observer blinded to treatment group as in our previous studies[10].

Spontaneous visceral pain assessment: The animals were placed individually in the observation chamber for a 25 min recording session. The observation chamber is a 28 cm × 17.5 cm × 12.5 cm see-through plastic home cage with one mirrored side located in an isolated room with constant "white noise". A digital camera located 0.5 meter from the chamber with an unobstructed view was used to record animals spontaneous visceral pain related behaviors. The camera was linked to a computer recording program for offline data analysis (Logitech Image Studio). The chamber was washed with a detergent disinfectant and dried after each use between animals. Postures defined as statistically significant increase in visceral pain-related behavior in this study included rearing, grooming and licking of the lower abdomen, stretching the abdomen or hindlimb, lowering the abdomen against the floor, and abdomen retractions or arching the back. Recordings were masked and analyzed by the investigator.

Necropsy and sample collection

Tissue collection: At the end of the one month experiment, animals were deeply anesthetized with isoflurane inhalation. Pancreatic, hepatic, gall bladder tissues were excised and a portion was fixed in cold 4% paraformaldehyde in 0.1 mol/L phosphate buffer saline (PBS). Thymus and splenic tissues were removed, weighed, and fixed in paraformaldehyde. Colonic tissues were removed and flushed with cold PBS, and portion of ascending and descending colon were fixed for histopathological examinations.

Histopathology

Hepatic and pancreatic samples were collected and immerse fixed overnight in 4% paraformaldehyde in 0.1 mol/L PBS, then transferred into 70% ethanol and embedded in paraffin. Sections were cut (5 μm), rehydrated, stained with hematoxylin and eosin for histopathological changes. In order to detect collagen fiber deposits, sections were further stained with Sirius Red (Electron Microscopy Sciences, #26357-02), using routine histological protocols[20].

Pancreatitis scores

Pancreatic tissues and a portion of the small intestine along with spleen were removed and processed for histopathological evaluations of the pancreatitis. The severity of lesions was scored on a 0-4 grade on the basis of the histopathological changes as follows: 0 - normal pancreatic microstructure, no inflammatory mononuclear cell infiltration; 1 - slight inflammatory mononuclear cell infiltration, with no detectable parenchymal destruction; 2 - mild pancreatitis, edema, focal parenchymal destruction with mononuclear cell infiltration; 3 - moderate pancreatitis, with diffuse parenchyma destruction, presence of necrosis, and reduced number of islets; and 4 - severe pancreatitis, parenchyma mostly destroyed and replaced with adipose tissues, loss of pancreatic islets, presence of fibrosis and or calculi.

Hepatitis score

A portion of the right lobe from liver tissues of each mouse was placed in an embedding cassette and fixed in paraformaldehyde as mentioned above. The specimens were dehydrated and embedded in paraffin, and tissue sections of 5 μm were stained with Hematoxylin Eosin. Each slide was evaluated under Ziess light microscopy. Hepatic lesions were graded on a scale of 0 to 4+ based on degeneration, inflammation, and necrosis as follow: Grade 0 - no detectable lesions, degeneration, infiltration of inflammatory cells, normal tissue appearance; Grade 1 - focal infiltration of inflammatory cells in the tissue and

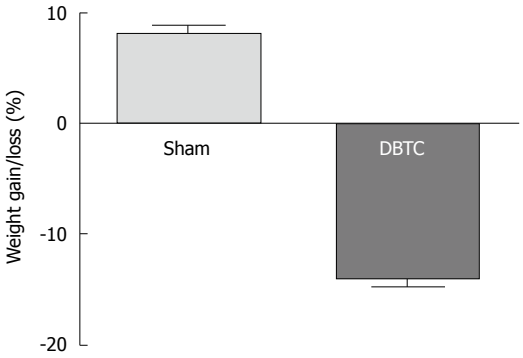

Figure 1 Percent weight gain in sham-treated controls (Sham) *vs* weight loss in dibutyltin dichloride-treated animals. *n* = 8 animals/group (*P* < 0.001). DBTC: Dibutyltin dichloride.

hepatocytes degeneration; Grade 2 - mild multifocal infiltration of inflammatory cells, and hepatocytes degeneration; Grade 3 - moderate multifocal infiltration of inflammatory cells and hepatocytes degeneration; and Grade 4 - severe diffuse infiltration of inflammatory cells, necrosis, or fibrosis.

Colitis evaluation scores

Colonic tissues were flushed with PBS (pH 7.2) and a portion from proximal and distal colonic tissues were fixed for histological examinations. The fixed sections were processed and stained with Hematoxylin Eosin and slides evaluated by Ziess light microscopy. The severity of colitis was assessed with a histological semi-quantitative grading score. The scores were based on histopathological features with a numeric value (0: normal to 4: severe) assigned according to the tissue involvement corresponding to the following criteria[21,22]. Grade 0: No detectable lesions, no inflammatory cells, and normal mucosal appearance; Grade 1: Focal inflammatory infiltrate in the mucosa; Grade 2: Mild multifocal inflammation with moderate expansion into the mucosa; Grade 3: Moderate multifocal inflammation with moderate expansion of the mucosa; and Grade 4: Severe diffuse inflammation with crypt epithelium disruption and ulceration.

Statistical analysis

All results are expressed as mean and standard error of mean (± SEM) unless otherwise stated. Data were analyzed using paired *t*-test comparison of groups for histology or analysis of variance (ANOVA) followed by Bonferroni post hoc comparison using GraphPad Prism Software for behavioral testing over time (San Diego, CA, United States). Statistical significance was set at $P \leqslant 0.05$.

RESULTS

Body weight loss

No major differences in body weight, behavioral analysis was detected between sham-treated and naïve control animals. Additionally, sham-treated and naïve control animals and wildtypes did not develop any histopathological lesions. Therefore, only sham-treated controls are reported here. Oral administration of DBTC resulted in weight lost as early as 3 d after treatment which persisted throughout the study, and animals developed persistent inflammatory abdominal and hindpaw hypersensitivity as compared to sham-treated animals. DBTC application induced significant body weight loss ($P < 0.001$) in compression to weight gain in sham-treated control animals. The major weight loss occurred during the 1st wk of DBTC inoculation when animals lost about 10% of their body weight, compared to weight gain in sham-treated animals. The body weight afterward became stable in DBTC-treated mice until the end of the one month study, but remained less than the sham-treated group (Figure 1).

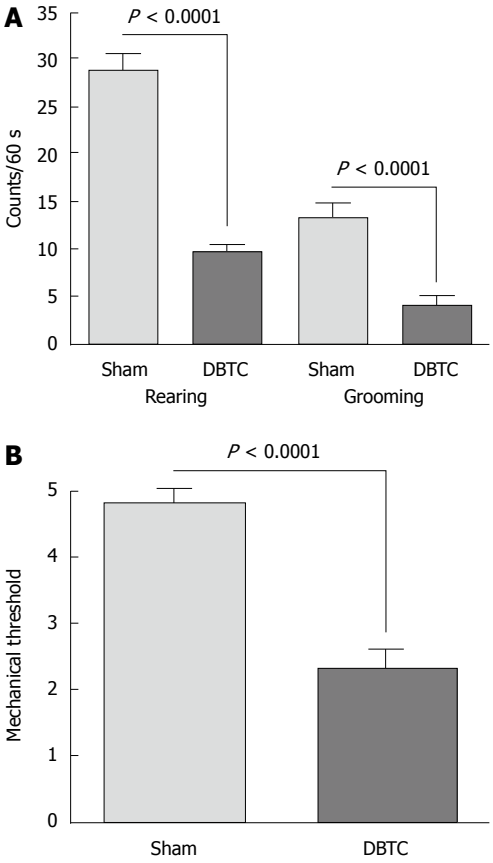

Figure 2 Rearing and grooming rate changes and hindpaw mechanical threshold modification due to dibutyltin dichloride-treatments. A: Rearing and grooming rates significantly diminished in dibutyltin dichloride (DBTC)-treated animals *vs* the sham-administered animals and persisted throughout the study (*P* < 0.0001); B: Hindpaw withdrawal threshold response to mechanical stimuli in DBTC-treated animals (DBTC) had significant decreased *vs* sham-administered control (sham) mice (*P* < 0.0001). *n* = 5 animals/group.

Behavioral pain related modifications

DBTC-treated animals had significant reduction in physical activities such as, cage crossing, rearing and grooming activity ($P < 0.0001$) compared to

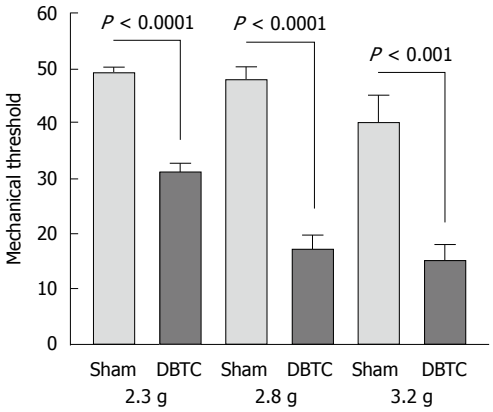

Figure 3 Abdominal threshold sensitivity to mechanical stimuli was determined with different gram forces of von Frey microfilaments as demonstrated here to 2.3 g ($P < 0.001$), 2.8 g ($P < 0.0001$), 3.2 g ($P < 0.001$), in dibutyltin dichloride-treated *vs* sham-treated controls (sham). Data is presented as the 50% mechanical threshold response. $n = 5$ animals/group.

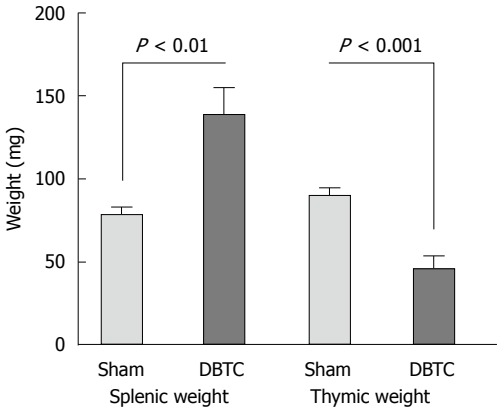

Figure 4 Splenic weight as well as splenic length (not shown) significantly increased in dibutyltin dichloride-treated animals ($P < 0.01$). In contrast thymic weight decreased in dibutyltin dichloride-treated (DBTC) *vs* the sham-treated (sham) animals ($P < 0.001$) due to atrophy and depletion of lymphocytes and thymocytes. $n = 8$ animals/group.

naïve sham-treated ones presumably attributed to the abdominal discomfort (Figure 2A). Hindpaw mechanical threshold was significantly decreased in DBTC-treated animals ($P < 0.0001$) tested using von Frey microfilaments (Figure 2B). In addition, responses to 3 different von Frey fibers with increasing grams force applied to the abdominal skin indicated DBTC-treated animals had significantly decreased mechanical threshold compared to sham-treated control (respectively $P < 0.0001$ from force 2.3 g, 2.8 g, $P < 0.001$ to force 3.22 g) (Figure 3). In contrast sham-treated animals demonstrated a partial visceral response to the higher filaments with forces of 3.22 g and above.

Splenomegaly and thymic degeneration

DBTC animals developed splenic hypertrophy with significant increase in weight and length of the spleens ($P < 0.01$). Splenic histopathologic studies demonstrated loss of medulla, irregular formation of trabecules, with captured trace of blirubin and bile deposits. In contrast, thymic tissues from DBTC-treated animals showed central degeneration and atrophy. The thymus was atrophied, and thymic weight significantly decreased in DBTC-treated animals ($P < 0.001$) compared to sham-treated animals (Figure 4).

Pancreatitis

Sham-treated animals demonstrated normal pancreatic structures with prominent islets (Figure 5A). In contrast, DBTC-treated animals developed gross as well as micropathology confirming the moderate to severe chronic pancreatitis. Pancreatic parenchyma presented with edema, congestion, distortion of microarchitecture, and infiltration of inflammatory cells. Pancreatic and acinar cells showed degeneration, fatty necrosis, and fibrosis. The pancreatic ducts became prominent and distended in DBTC-treated animals. These findings were consistent with fibrotic thickening which were particularly prominent in the vicinity of the primary duct, as well in surrounding lobular pancreatic parenchyma as confirmed with Sirius Red histopathological studies.

Also noted was loss of microstructure indicated by the presence of irregular and degenerated islets, along with vacuolization and necrosis of β cells accompanied by invasion of inflammatory cells. The sizes as well as the numbers of the pancreatic islets were significantly diminished in DBTC-treated compared with the sham-treated animals (Figure 5B). A few small and shrunken islets were scattered throughout the pancreatic parenchyma, but overall loss of β cells was evident. In addition, pancreatic ducts had become thickened and expanded containing traces of debris and calculi formation (Figure 6A). Extent of pancreatic damage scored (0- normal to 4 most severe) were 3.6 ± 0.4 (severe) in DBTC-treated in contrast to score 0 (normal) for sham-treated animals.

Gall bladder

Gall bladder showed extensive expansion with ductal distension and occasional detected bile stones (Figure 6B).

Hepatitis

Hepatic tissues became enlarged, friable and pale or yellowish in color with a spotted appearance indicating moderately severe hepatitis. Macroscopic hepatic injuries were evidenced with activation of stellate cells, degeneration of hepatocytes, and multifocal and central necrosis (Figure 7A). Additionally, hepatic structure showed fatty degeneration of hepatocytes, and periportal infiltration of inflammatory cells, along with presence of visible ductal dilation. Fibrotic thickening was prominent in the vicinity of portal ducts, and surrounding lobular hepatic parenchyma as confirmed with Sirius Red histopathological studies. The severity of hepatitis was scored 3.7 ± 0.2 (severe) in DBTC-treated animals compared to the score 0

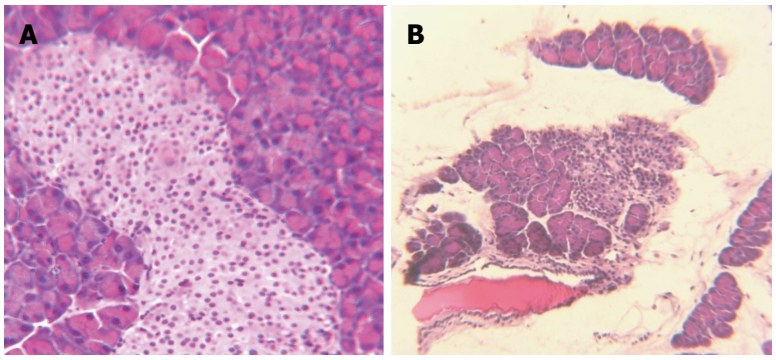

Figure 5 Pancreatic photomicrographs from sham-treated normal control (A) compared to dibutyltin dichloride-treated animal (B). A: Pancreas: Photomicrograph demonstrates sham-treated control pancreas with normal pancreatic microstructure and islet cells. Representative slide from *n* = 8 animals/group; B: Pancreatitis: Photomicrograph illustrates pancreas from a dibutyltin dichloride-treated animal with significant loss of pancreatic structure and islets, acinar atrophy, infiltration of inflammatory cells, fatty deposits and edema (magnification × 40). Representative slide from *n* = 8 animals/group.

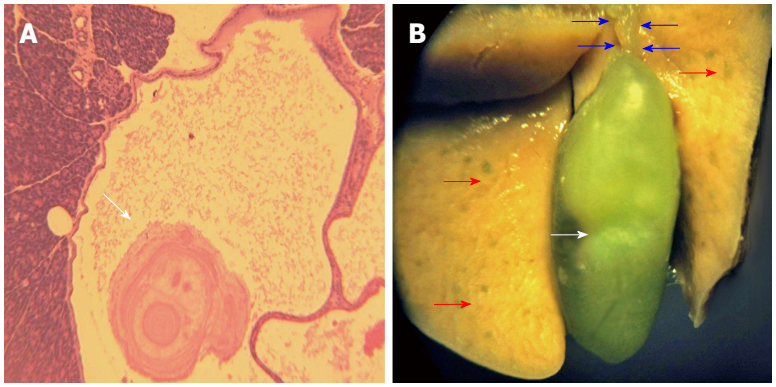

Figure 6 Photomicrograph from pancreatic ductal calculi formation (A), as well as hepatic and gall bladder gross pathological structure (B) in dibutyltin dichloride-treated animals. A: Calculi formation: Photomicrograph illustrates histopathologic structure from a representative pancreas of a dibutyltin dichloride-treated animal to demonstrate ductal distension and calculi formation. Representative slide from *n* = 8 animals/group (magnification × 40); B: Photograph illustrates hepatic gross lesions (red arrows), expanded bile duct (blue arrows) and gall bladder (white arrow) from DBTC-treated animal. Representative from *n* = 8 animals/group.

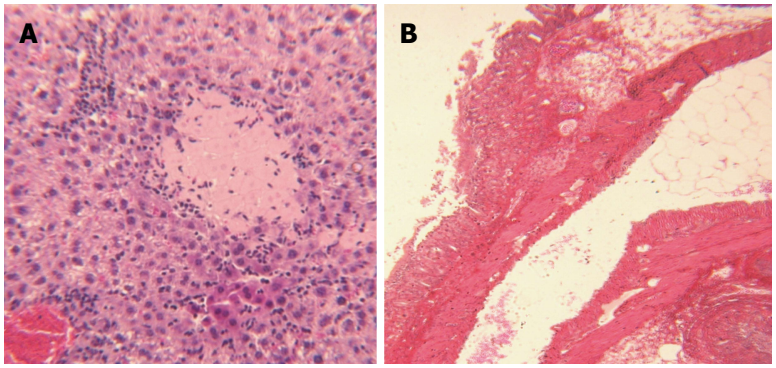

Figure 7 Photomicrograph of hepatitis (A) and colitis (B) from dibutyltin dichloride-treated animals. A: Hepatitis: Photomicrograph illustrates necrosis of hepatocytes, infiltration of inflammatory cells (magnification × 40). Representative slide from *n* = 8 animals/group; B: Colitis: Photomicrograph demonstrates distortion of colonic microstructure, loss of brush boarder epithelial cells, infiltration of inflammatory cells into mucosa and cryptic abscess formation. Representative slide from *n* = 8 animals/group.

(normal) in sham-treated animals.

Colitis

Colonic tissue showed extensive necrosis and loss of intestinal epithelial cells, distortion of cryptic structures, thickening of mucosa due to invasion of inflammatory cells, and some cryptic microabscess formation, presenting advanced colitis (Figure 7B). Colonic lesions and colitis in DBTC-treated animals were scored 3.4 ± 0.3 (moderately severe) compared to 0 (normal) for

the sham-treated animals.

DISCUSSION

Here our findings may provide a new model to better approach investigation of chronic multiorgan inflammatory and visceral pain in murine model and to study possible potential targets in this model for the treatment of chronic hepatobiliary and pancreatitis. Acute pancreatitis is manifested by histopathological transformations, including the presence of inflammatory mediators, acinar atrophy, fat necrosis, intraductal hemorrhage, and stromal proliferation[23]. Chronic pancreatitis is distinguished by recurrent or continuous inflammation of pancreatic progressive atrophy and irreversible fibrosis, with demise of exocrine and endocrine malfunction in severe forms. While, severe and uninhibited abdominal pain is the main aspect of persistent pancreatitis, the mechanism/s by which the pain is induced is poorly explored possibly due to a lack of available appropriate animal model to mimic chronic pancreatitis[24]. In current study the TNFR1/R2 deficient animals displayed significant pain related modifications such as decreases in mechanical threshold after DBTC treatment that persisted through the one month experiment until the end of the study. The significant decreases in mechanical threshold were detected on foot pads and abdomen after the induction of the injury as compared with sham treated animals.

Although, chronic pancreatitis in human is distinguished by irreversible fibrosis, yet pancreatic fibrosis in animal models are mainly reversible[25]. Indeed persistent fibrosis in vital organs results in significant morbidity and mortality worldwide. While, organ fibrogenesis is typically the end product of various non-resolving or repetitive injuries such as chronic infection and radiation exposure; abnormal repair reaction followed by tissue injuries to contribute to the progression of organ fibrosis. Indeed, fibrogenesis is required in natural wound healing process, persistent fibrogenesis in organs can lead to devastating symptoms and organ failure[1,2]. At the cellular level fibrogenesis is remarkably similar progress in different organs and can cause generalized fibrosis in these tissues. Yet, currently there is no appropriate model available to study systemic fibrosis.

Similarly chronic hepatitis and hepatic fibrosis result from excess extracellular matrix produced primarily by hepatic stellate cells. We and other investigators have shown that proinflammatory cytokines (*e.g.,* TNFα) and other inflammatory mediators such as growth factors are regulated by matrix metalloproteinase (MMPs) expressions[20,26]. Further activation of Stellate cells is major event in hepatic fibrosis formation caused by multiple injuries due to chemicals, infectious agents, surgical and/or inflammatory cytokine and chemokines which prompt proliferation and transformation of stellate cells to secret extra cellular matrix. Additionally, the mesenchyme-specific transcription factor forkhead box f1 (Foxf1) in liver is specifically expressed in hepatic stellate cells. Recently, a lipid based liver-specific delivery system also called "dbtc" is reported to be efficient to transfer the Foxf1 siRNA to activated hepatic stellate cells and silence genes expressed in different cell types in liver when used in an acute mouse model of bile duct ligation-induced secondary cholestasis[27].

Pancreatitis models are divided into surgically induced and chemical administration induced hypersecretion of the pancreatic enzymes. The surgical models include ligation and/or cannulation of the biliopancreatic ducts with infusion of variety of solutions, or the formation of closed duodenal loops. Pancreatic fibrosis in bile duct ligated rats is a difficult model to induce and requires increased stimulation. Chemical secretagogues (caerulein or l-arginine) include administration of DBTC to cause a partial blockage of the pancreatic ducts to induce pancreatic disease through enzymic reflux into the gland[28].

Various environmental chemicals have been implicated in the induction of autoimmune responses. Di-n-dibutyltin dichloride is an organotin compound and PVC plastic additive that frequently released to contaminate food and water. As eventually DBTC is degraded in the environment with possible harmful effects on man and animals[29]. Some therapeutic indications of DBTC include at a dose of 10 mg/kg per day for 5 consecutive days effective to eliminate *Trypanosoma brucei* infection in mice[23]. LD50 of DBTC is reported to be 90 mg/kg[30]. Metabolism of DBTC by cytochrome P450 enzymes plays an important role in the induction of biological effects, as DBTC with affinity for mitochondria depresses respiration and elevates serum enzymatic activities resulting in hepatic injuries[31]. Thymus atrophy noted in the current investigation was similar to that reported as a consequence rather than a cause in DBTC-intraperitoneal injected rats[32]. Increased proliferin expression and promotion of morphological thymic transformation reportedly occurring at similar concentrations most probably are DBTC-induced thymus involution. Indeed, this reaction is due to antiproliferative activity of DBTC, as observed by inhibition of thymidine incorporation of thymocytes isolated from DBTC-treated rats[32]. After administration of 4-61 mg/kg iv or 120-240 mg/kg oral DBTC, a dose dependent reversible reduction of thymus weight and number of thymocytes were observed in mice. Iv administration of DBTC highly increased the level of total bilirubin in serum of these animals. But, the level of bilirubin in serum did not correlate with the thymotoxic effects of DBTC in mice[33]. Of interest, toxicity of DBTC in mice is reported after 3 consecutive daily high doses of 50 mg/kg, killing 75% and the survivors developed severe hepatic and bile duct damage. While 3 daily doses of 20 mg/kg caused only mild bile duct and liver lesions[34].

In another study, mice were given DBTC at 8, 15, or 30 mg/kg per day by gavage on days 0-3 or days 4-7 and sacrificed on day 18 of pregnancy. The incidence of embryonic loss increased on days 0-3 at 15 mg or over and, on days 4-7 with 8 mg/kg bw/d and higher. However, no increase in the rate of fetus malformations was observed after the DBTC administration. A decline in the serum progesterone levels was noted in dams given DBTC at 30 mg/kg per day, which might have affected the pregnancy initiation, maintenance, and loss when administered during early pregnancy[35].

Non-alcoholic steatohepatitis (NASH), the most common hepatic disorder, is manifested with inflammation, hepatocyte injury, cell death, fibrosis and multiorganelle failure, leading to cirrhosis[26]. Previously we reported cytokine/chemokine, extracellular matrix accumulation and metalloproteinase upregulation in a dietary deficient NASH model[20]. RT-PCR measurements showed a significant overexpression of inflammatory cytokines [TNFα, transforming growth factor (TGF-β), interleukin (IL-1β), IL-6], suppressor of cytokines signaling1 and genes involved in tissue remodeling and fibrosis (MMPs, collagen-α1) in the hepatic tissues of rats fed methionine-choline deficient diet[20].

Furthermore, using DBTC tail injection rat model we have shown implication of the endothelin cascade gene expression as a major contributing factor in pancreatic pain in both pancreatitis and potential pancreatic cancer[6]. In the present study oral inoculation of DBTC in TNFR1/R2 deficient mice induced a chronic persistent multiorgan hepatobiliary pancreatitis as confirmed by pathological studies including biliary dilation, loss of hepatic and pancreatic architecture and islets, edema in parenchyma, infiltration of inflammatory cells, degeneration, vacuolization and fibrosis, and pancreatic necrosis of acinar cells. Pain related behaviors were increased in animals with pancreatic inflammation including visceral pain-related behavior and secondary cutaneous mechanical hypersensitivity which increased greater than 2-fold. Here lack of TNF receptors appears to accelerate the inflammatory response in multiple organs and contribute to fibrosis in hepatobiliary and pancreatic tissues. A serum proteome profiling analysis in our previous study in TNFR1/R2 deficient mice with pain related behaviors in an arthritis model revealed high levels of serum inflammatory factors. The inflammatory factors included TNFα, which is regulated by the activation of normally T-cell expressed and secreted (RANTES), chemokine (C-X-C motif) ligand 9 [CXCL9 (MIG)], chemokine (C-X-C motif) ligand 10 [CXCL10 (IP-10)], and chemokine (C-C motif) ligand 2 [CCL2 (MCP-1)][10].

Primary sclerosing cholangitis is a complex hepatic disorder, characterized by chronic inflammation of the biliary epithelium, and cholestasis resulting in multifocal bile duct strictures, fibrosis of hepatic parenchyma and biliary tract leading to cirrhosis and malignancy. The etiology of primary sclerosing cholangitis is not fully discovered and no effective therapy is available[5]. Gallstone, one of the most prevalent gastrointestinal complications, is multifactoral, with serious outcomes such as acute gallstone pancreatitis and gallbladder cancer. Gallstone disease is a chronic recurrent hepatobiliary complication which is manifested by creation of gallstones in the hepatic and bile duct, or gallbladder. It is manifested by dysfunctional metabolism of cholesterol, bilirubin and bile acids[7]. Other factors may involve genetic, environmental and steroids. The prevalence of gallstone disease has increased because of sedentary lifestyle and poor diets. Gallstones are known as a common cause of pancreatitis. From 932 patients with acute pancreatitis 40% had gallstones, and 22% alcohol induced[36]. Further, pancreatitis is frequent amongst IBD patients. Gallstones are reported as the most frequent cause of pancreatitis in IBD patients which cause growing diagnostic challenges[37]. Thus, this model may facilitate study of fibrogenesis and/or fibrosis resolution in multiple vital organs leading to development of novel technologies and therapeutic strategies aimed at lessening organ fibrosis.

In conclusions, this is the first report of a chronic inflammatory multiorgan hepatobiliary pancreatitis along with fibrosis and calculi formation model that can be induced reliably with use of oral DBTC administration in TNFR1/R2 deficient mice. Future studies will utilize this model in investigations of anti-fibrotic and analgesic therapeutics.

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COMMENTS

Background

Chronic multiorgan, pancreatitis and hepatobiliary complications manifested with irreversible fibrosis and spontaneous visceral pain in patients. Currently there is no cure or reliable model available for chronic pancreatitis or multiorgan fibrosis other than repeated dosing with chemical caerulein to produce acute flares. An appropriate murine model to mimic the syndrome is desirable. Pancreatic fibrosis in bile duct ligated rats is a difficult model to induce and requires other increased stimulations. The prevalence of gallstone disease has increased because of sedentary lifestyle and poor diets. Dibutyltin dichloride (DBTC) is a biocide, and antifouling agent in the paint and fabric industry. Tail vein injection of DBTC induces unpredictable pancreatitis flares in rats, DBTC injection is tedious, and minor leakage results in tail gangrene and animal loss. TNFα proinflammatory cytokine initiates inflammation through its 2 receptors, TNFR1/R2. We devised a new chronic model of pancreatitis and multiorgan

inflammation in TNFR1/R2 deficient mice using oral DBTC.

Research frontiers

Currently, there is no cure or reliable model available for chronic pancreatitis and multiorgan fibrosis in mice. Persistent pancreatitis manifests with severe abdominal pain, but the mechanism/s by which induced is/are poorly explored possibly due to lack of appropriate models. Three daily doses of 20 mg/kg DBTC caused only mild bile duct and liver lesions, while 3 consecutive daily doses of 50 mg/kg DBTC were toxic and killed 75% of mice. TNFα proinflammatory cytokine initiates inflammation through its 2 receptors, TNFR1/R2. Proteome profiling analysis in our previous study in TNFR1/R2 deficient mice with persistent pain related behaviors in an arthritis model revealed high levels of serum inflammatory cytokines likely responsible for the multiorgan inflammatory response in this model.

Innovations and breakthroughs

This is the first report of a chronic inflammatory hepatobiliary pancreatitis, colitis and stone formation model that can be induced reliably with use of oral DBTC-administration in TNFR1/R2 deficient mice. These findings provide this new murine model to better approach investigation of chronic multiorgan inflammatory and visceral pain in murine mode. In addition, to facilitate study of fibrogenesis and/or fibrosis resolution in multiple vital organs leading to development of novel technologies and therapeutic strategies aimed at lessening organ fibrosis.

Applications

This chronic inflammatory hepatobiliary pancreatitis, colitis/fibrosis and stone formation model can be induced reliably utilizing oral DBTC in TNFR1/R2 deficient mice. The model can be used to investigate chronic multiorgan inflammatory and visceral pain in mice to explore the mechanisms of injury and to study of fibrogenesis and/or fibrosis resolution in multiple vital organs leading to development of novel technologies and therapeutic strategies aimed at lessening organ fibrosis. This study grants ability for further investigation into the use of this model to explore mechanisms of multiorgan injury, the biochemical players and the therapeutic exploration for devastating chronic multiorgn inflammatory and fibrogenesis such as cystic fibrosis and systemic sclerosis as well as cholangitis.

Terminology

TNFR1/R2 deficient mice are transgenic animals lacking tumor necrosis factor receptor 1 and 2 with constant higher proinflammatory TNFα levels compared to wildtype background animals. Multiorgan damage, when 2 or more organs involved in the course of injury. Fibrogenesis is a process usually followed chronic inflammatory to form fibrous structures in and around tissues. Pain related mechanical hypersensitivity, is measured by von Frey microfilament (an accepted standard procedure), demonstrating decreased tolerance to a simple touch manifested with withdraw to protect against induced excess pressure while normal animals tolerate the mechanical touch with no withdrawal response to the fine microfilament touch.

Peer-review

This is an interesting and well-written paper. The author provided that TNFR1/R2 deficient mice treated with DBTC reveal the severe chronic injury of various internal organs which was proved with usage of histological methods.

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ORIGINAL ARTICLE

Basic Study

Phosalone-induced inflammation and oxidative stress in the colon: Evaluation and treatment

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Author contributions: Ghasemi-Niri SF performed the majority of experiments and analyzed the data; Baeeri M performed the molecular investigations; Gholami M participated in treatment of animals; Ghasemi-Niri SF, Maqbool F and Abdollahi M designed, coordinated the research and wrote the paper; and Abdollahi M conceived the study.

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Abstract

AIM: To investigate the side effects of phosalone on intestinal cells and to evaluate benefits of ellagic acid (EA) as a remedy.

METHODS: In order to conduct an *in vivo* study, a rat model was used. The rats were divided into ten groups based on the materials used in the experiment and their dosage. The first group was fed normally. The second group was administered EA through gavage. Next Four groups were given (1/3, 1/5, 1/10, 1/20) LD_{50} phosalone; an organophosphorus compound. The last four groups received (1/3, 1/5, 1/10, 1/20) LD_{50} phosalone and of EA. After one month, the rats were sacrificed and their colon cells were examined to evaluate the level of inflammation, proteins and oxidative stress markers.

RESULTS: The results of this research show that phosalone elevates oxidative stress and changes the level of tumor necrosis factor-a (TNF-α), interlukin-

6β (IL-6β) and nuclear factor (NF)-κB proteins. EA administration reduced phosalone toxicity and changed oxidative stress and inflammatory markers for all phosalone doses. Overall changes in reduction of TNF-α (230.47 ± 16.55 pg/mg protein *vs* 546.43 ± 45.24 pg/mg protein, $P < 0.001$), IL-6β (15.85 ± 1.03 pg/mg protein *vs* 21.55 ± 1.3 pg/mg protein, $P < 0.05$), and NF-κB (32.47 ± 4.85 pg/mg protein *vs* 51.41 ± 0.71 pg/mg protein, $P < 0.05$) manifest that the efficacy of EA is more viable for 1/3 LD_{50} dose of phosalone. Furthermore, EA is effective to counteract the negative outcomes of oxidative stress. When EA was used to treat 1/3 LD_{50} of phosalone's side effects, it improved the level of AChE activity (48.5% ± 6% *vs* 25% ± 7%, $P < 0.05$), TTM (0.391 ± 0.008 mmol/L *vs* 0.249 ± 0.032 mmol/L, $P < 0.05$), FRAP (46.04 ± 5.005 μmol/L *vs* 18.22 ± 1.9 μmol/L, $P < 0.01$) and MPO (0.222 ± 0.019 U/mg protein *vs* 0.387 ± 0.04 U/mg protein, $P < 0.05$).

CONCLUSION: This research highlights that EA is effective to alleviate the side effects of phosalone by reducing the level of oxidative stress and inflammatory proteins.

Key words: Organophosphorus; Phosalone; Ellagic acid; Inflammation; Oxidative stress; Colon

Core tip: This research uses a rat model to evaluate the colon related side effects of phosalone which is a member of the organophosphorus family. After feeding different dosages of phosalone to the rats for one month, the colon tissue of the rats were studied using oxidative stress and pathology tests. Both tests show that the higher doses of phosalone elevate reactive oxygen species (ROS), tumor necrosis factor-α, interlukin-6β and nuclear factor-κB proteins which result in more inflammation. In our study, ellagic acid (EA) which is a strong antioxidant reduced phosalone-induced side effects. The oxidative stress and pathology results concluded that EA helps reducing inflammation and ROS.

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INTRODUCTION

Pesticides are substances used in agriculture to kill pests and also as a domestic insect killer[1]. Although they are significant in agriculture use but they may also enter human body through inhalation *via* air born particles. Farmers may inhale such chemicals when they use them for pest control[2]. General public is prone to pesticide after eating agricultural products which are not washed properly. Over-usage of pesticides may cause plants to absorb them directly or indirectly through soil. In such case, washing may not completely cleanse the pesticides and their consumers are vulnerable to the resultant side effects[3,4].

Phosalone [O,Odiethyl-S-(6-chloro-2-oxobenzoxazolin-3-yl-methyl)-phosphorodithioate] is a member of the organophosphorus (OP) family, which is used extensively as a pesticide in agriculture and as a domestic insect killer[5]. As compared to Dicoloro Di Three ethane (DDT), phosalone has less severe side effects on human and environment and because of this reason it has replaced DDT for pest control. Regardless of the fact, that phosalone is safer than DDT, but its toxicity has been one of the important research topics in toxicology. The most important known toxicity of phosalone is related to human nervous system. The mechanism of such damage is extremely toxic and phosalone can inhibit neural cholinesterase (ChE) activity, which elevates the level of acetylcholine thus therefore prevents neural signal pathway in the nervous system[6]. Furthermore, like the other members of the OP family, phosalone increases reactive oxygen species (ROS) in the human body tissues thus reduces the level and activity of anti-oxidant enzymes. Higher amount of ROS increases lipid peroxidation (LPO) in the membrane of cells, resulting in membrane damage and disturbance in the cell functional balance[7]. The final repercussions of ROS are faster cell aging and higher chances of DNA and RNA changes, subsequently leading toward cancer and gene mutations[8,9].

The main route through which OP enters the body is mucosa in intestinal cells, where OP can pass through membrane barrier and enter blood. Human cardiovascular system distributes OP to other organs and results in nervous system and ROS related damages[10,11]. Furthermore, the effect of OP on micro flora in intestinal and gastrointestinal enzymes elevate neutrophil infiltration and pro-inflammatory proteins[12,13]. The consequence of such effects is the migration of several immune cells such as neutrophils, monocytes, lymphocytes, macrophages and chemokines then adhesion molecules move toward mucosal tissue. The final outcome of such damage is intestinal inflammation[14,15].

This research elaborates ROS related side effects of phosalone and proposes a material to reduce and potentially eliminate such side effects. The proposed material should be able to offset free-radicals. This research shows that ellagic acid (EA) can be a remarkable candidate to considerably suppress the side effects of phosalone. EA is an important natural occurring substance, which has phenol components[16]. EA is present in numerous fruits and vegetables such

as grapes, nuts, strawberries, black currents, raspberries, green tea, pomegranates, and the stem and bark of Eucalyptus globulus, Eucalyptus maculatu and nuts. The international chemical name of EA is 2,3,7,8-tetrahydroxy-chromeno[5,4,3-cde] chromene-5,10-dione[17].

The biological activities of EA has been investigated in several *in vivo* and *in vitro* studies and have shown that EA has anti-cancer, anti-inflammatory and anti-oxidant properties and in addition it has beneficial therapeutic effect on colon, skin, breast cancer and inflammatory bowel disease (IBD)[18]. EA can also improve mucosa production in goblet cells in colon; reduce pro-inflammatory proteins COX-2 and iNOS over expression and neutrophil infiltration[19]. The anti-oxidant effect of EA stem is clear from the fact, that EA can scavenge free radical, nitrogen reactive species, and ROS, including hydroxyl radicals, peroxyl radicals, NO_2 radicals, and peroxynitrite and therefore EA reduce DNA and cell damages[20]. Additionally, EA can potentially shield DNA and protect it from ROS, free radical and chelation of metal ions attack.

Regarding other effects of EA, some studies have reported that EA can affect cytochrome C in mitochondria which increases BAX/Bcl2, regulates cell division and apoptosis[21]. Also through stimulating the immune system, EA plays a positive role in intercellular complex signaling systems such as mitogen activated protein kinases (MAPKs) and/or the transcription factor nuclear factor κB (NF-κB)[22]. An in-depth study of these effects is presented in this paper.

In our study, we evaluate effect of phosalone on inflammation and oxidative stress with four doses as well as subsequent effect of EA on colon cells.

MATERIALS AND METHODS

Chemicals

Acetylthiocholine iodide, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) from Merck (Germany), trichloroacetic acid (TCA), Tris base, 1,1,3,3′-tetraethoxypropane (MDA), 2-thiobarbituric acid (TBA), *n*-butanol, 2,4,6-tripyridyl-*s*-triazine (TPTZ), n-butanol, acetic acid, $FeCl_3$-$6H_2O$, benzethonium chloride, 5,5′-Dithiobis(2-nitrobenzoic acid), Trizma® base, EA, o-Dianisidine dihydrochloride, phosphate buffer from Sigma-Aldrich (Germany), n-butanol, hexadecyl tri-methyl ammonium bromide (HETAB), ethylene diamine tetra acetic acid (EDTA), hydrochloric acid (HCL), acetic acid, sodium acetate, hydrogen peroxide (H_2O_2), O-dianisidine hydrochloride, ferric chloride ($FeCl_3$-$6H_2O$), Coomassie reagent, bovine serum albumin (BSA), sodium sulphate (Na_2SO_4), sulphuric acid (H_2SO_4), phosphoric acid (H_3PO_4), potassium dihydrogen phosphate (KH_2PO_4), potassium hydrogen diphosphate (K_2HPO_4), sodium carbonate (Na_2CO_3), cupric sulphate ($CuSO_4$-$5H_2O$) from Merck. Rat-specific tumor necrosis factor-α (TNF-α), interlukin-6 (IL-6) and NF-κB ELISA kits from (Bender MedSystems GmbH, Austria), analytical grade form of phosalone from local pesticide manufacturing companies (Agroxir) and were used in this study.

Experimental animals

In our study, male Wistar rats weighing 180-200 g were selected according to the regulations of the ethics committee of Tehran University of Medical Sciences (TUMS) approved with code number of 93-02-45-26666. Animals were housed separately in standard polypropylene cages with a wire mesh top, kept under standard conditions, including temperature (23 ℃ ± 1 ℃), relative humidity (55% ± 10%) and 12/12 h light/dark cycle, and fed a standard pellet diet and water ad libitum. All ethical themes of studies on animals were considered carefully.

Experiment design

Animals were divided into ten groups based on the materials used in the experiment and their dosage, with six rats in each group. The first group was fed normally. The second group was administered EA (10 mL/kg) through gavage. Next Four groups were given different dosage of phosalone (1/3 LD_{50}: 40 mg/kg, 1/5 LD_{50}: 20 mg/kg, 1/10 LD_{50}: 12 mg/kg and 1/20 LD_{50}: 6 mg/kg), which is a member of organophosphorus family, through gavage. The last four groups received both phosalone (1/3 LD_{50}: 40 mg/kg, 1/5 LD_{50}: 20 mg/kg, 1/10 LD_{50}: 12 mg/kg and 1/20 LD_{50}: 6 mg/kg) and EA (10 mL/kg). After one month, the rats were sacrificed and their colon cells were examined to evaluate the level of oxidative stress factors.

Sample preparation

After 30 d, all rats were anesthetized (40% Ketamine 1000, 25% Xylazine 2%, 0.1 mL/100 g body weight) and after that all of animals were humanly sacrificed and colonic tissues were immediately separated. Isolated segments were rinsed with normal saline and then placed in an ice bath throughout the procedure. Colonic tissue was divided into two pieces. The first piece was weighed and kept in 10 mL of formalin 10%, as a fixator for the purpose of histopathological evaluation. The second piece was weighed and homogenized in 10 volumes of ice cold potassium phosphate buffer (50 mmol, pH = 7.4) and then stored at -20 ℃ for 24 h. The sample was then sonicated and centrifuged for 30 min at 3500 *g*, and the supernatant was transferred to a micro tube. Then sample was kept at -80 ℃ until biomarker analyses.

Determination of lethal dose of phosalone

An lethal dose (LD_{50}) of phosalone is a standard measurement of toxicity that is stated in milligrams (mg) of phosalone per kilogram (kg) of body weight at which 50% of rats are killed. For finding the LD_{50} of phosalone, we performed a study on Wistar rats.

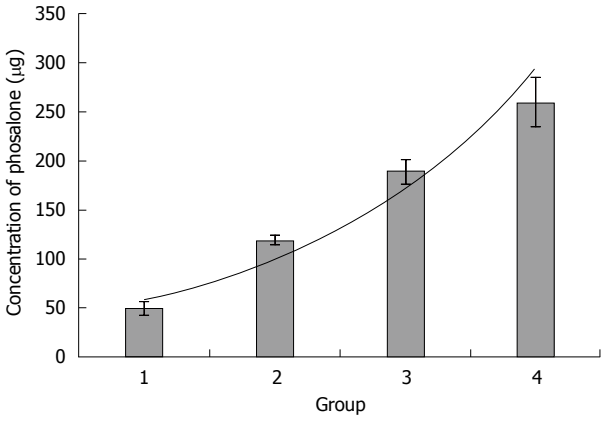


Figure 1 Determination of LD_{50} of phosalone.

We divided five groups of rats and administrated with different doses of phosalone. One of group was control that didn't receive phosalone. But four groups received different doses of phosalone, like 50 mg/kg, 120 mg/kg, 190 mg/kg and 260 mg/kg. After two days we compared all groups and found LD_{50} was between 120 mg/kg to 190 mg/kg. After that we analyzed all data and 120 mg/kg was LD_{50}, used for phosalone in animal model in our study (Figure 1).

Assay of oxidative stress enzymes

AChE activity: AChE activity of erythrocytes was measured according to method of Ellman method using acetylthiocholine iodide as the substrate and 5-5-bis dithionitrobenzoic acid (DTNB). Briefly, 10 μL of sample was added to 3 mL of solution containing 25 mmol/L DTNB in 75 mmol/L phosphate buffer. Then 10 μL of 3 mmol/L acetylcholine iodide was added and absorbance changes were measured at 412 nm in a two-fold rays spectrophotometer[23].

Myeloperoxidase activity assessment

MPO activity was determined by a dianisidine-H_2O_2 method, modified for 96-well plates. Briefly, plasma samples (10 μg protein) were added in triplicate to 0.53 mmol/L o-dianisidine dihydrochloride (Sigma) and 0.15 mmol/L H_2O_2 in 50 mmol/L potassium phosphate buffer (pH 6.0). After incubation for 5 min at room temperature, the reaction was stopped with 30% sodium azide. The absorbance was measured at 460 nm (ε = 11300 $M^{-1}\cdot cm^{-1}$) spectrophotometrically (Shimadzu 160A UV-VIS spectrophotometer). Results were expressed as units of MPO/mg protein, whereby 1 unit of MPO was defined as the amount of enzyme degrading 1 nmol H_2O_2 per min at 25 ℃[24].

LPO measurement

To measure LPO, thiobarbituric acid-reaction substances (TBARS) were assessed in colon tissue. TBA reacts with lipid peroxides in the samples producing a measurable pink color that has absorbance at 532 nm by a double beam spectrophotometer. Concentration of TBARS is recorded as μg[25].

Assay of total thiols

To determine TTM in the control and test groups, 0.6 mL Tris-EDTA buffer (Tris base 0.25 mol/L, ethylene diamine tetra acetic acid 20 mmol/L, pH 8.2) was added to 0.2 mL of supernatant, and after quick vortex mixing, 40 μL 5-5'-dithiobis-2-nitrobenzoic acid (10 mmol/L in pure methanol) was added. The final volume of this mixture was made up to 4.0 mL by an extra addition of pure methanol. After 15 min incubation at room temperature, the samples were centrifuged at 3000 *g* for 10 min and ultimately the absorbance of the supernatant was measured at 412 nm. Data are shown as mmol/L[26].

FRAP assay

Antioxidant power of plasma was evaluated by measuring its ability to reduce of Fe^{3+} tripyridyltriazine (TPTZ) complex (colorless) to Fe^{2+} TPTZ (blue colored) formed by the action of electron donating antioxidants at low pH. The ferric reducing antioxidant power (FRAP) reagent was prepared by mixing 300 mmol/L acetate buffer, 10 mL TPTZ in 40 mmol/L HCl and 20 mmol/L $FeCl_3$ in the proportion of 10:1:1 at 37 ℃. 10 μL of the H_2O diluted sample was then added to 300 mL freshly prepared reagent warmed at 37 ℃. An intense blue color complex was formed when Fe^{3+} TPTZ complex was reduced to Fe^{2+} form. The complex between Fe^{2+} and TPTZ gives a blue color with absorbance at 593 nm. Data are shown as μmol/L[27].

Determination of TNF-α and IL-6β

A human specific ELISA kit (BenderMed System) was used to quantify TNF-α and IL-6 in the supernatant of colon tissue. To assess the amount of TNF-α, the absorbance of sample was measured in 450 nm as the primary wavelength and 620 nm as the reference wavelength by ELIZA reader as described in the kit brochure. TNF-α and IL-6β levels were expressed as pg/mg protein of tissue[28].

Determination of NF-κB

The amount of NF-κB in colon cells extracts was measured by using NF-κB ELISA kits (BenderMed System) according to the manufacturer's instructions. The levels of NF-κB in nuclear extracts were calculated using the standard curve and expressed as pg/mg protein[29].

Total protein assessment

The concentration of protein in the colon homogenate was measured by the Bradford method using BSA as the standard. The absorbance was measured by the spectrophotometer at 595 nm after 5 min. The bovine serum albumin was used as standard[30].

Statistical analysis

At least four independent experiments in repetition were carried away. Data are presented as mean ± SE. One-way ANOVA and Tukey's multi-comparison trials were held out by Stats-Direct 3.0.169 software to determine the statistical differences while the degree of significance had been set at *(P* < 0.05*)*.

RESULTS

Pathology evaluation of the colon damage

As shown in Figure 2, histopathological examination in normal group shows that there was no ulceration, no necrosis, no adhesions, no wall thickening and mucosal/submucosal polymorphonuclear (PMN) leukocyte infiltration. In EA group there was no blood and ulcer in mucosal/submucosal of the colon tissue. In the 1/3 LD_{50} phosalone group, it was observed in some areas infiltration, adhesions, with no any overlying blood and serous adhesion.

The 1/3 LD_{50} phosalone and EA group showed improvement in muscles and mucosa, a reduction inflammation in colon tissue and low lymphocytes infiltration in submucosal layer. The mucosal glands are normal but mild degeneration of mucosal muscle cells and muscle layers is observable. The level of degeneration and inflammation is less than 1/3 LD_{50} phosalone group. Histological examination of 1/5 LD_{50} phosalone and EA group showed improvement in mucosa with the reduction lymphocytes in submucosa region. The mucosal glands are normal but mild degeneration of mucosal muscle cells and muscle layers is observable. The level of degeneration is less than 1/5 LD_{50} phosalone group. In the 1/10 LD_{50} phosalone and 1/20 LD_{50} phosalone with EA groups, the mild degeneration of mucosal muscle cells and muscle layers were observed. In 1/10 LD_{50} phosalone and EA was seen a very mild inflammation due to lymphocytes infiltration between mucosal glands. But in 1/20 LD_{50} phosalone and EA, there was no inflammation in different layers.

AChE activity

After pathological examination, the first step was the evaluation of EA through measurement of AChE activity. AChE activity was reduced in colon cells of groups receiving 1/3 and 1/5 LD_{50} of phosalone in comparison to normal group ($P < 0.01$). In two groups (1/3 and 1/5) LD_{50} phosalone, AChE activity was significantly decreased in comparison to EA group ($P < 0.05$). EA restored the activity of AChE which was suppressed by phosalone. Among different phosalone doses, such AChE activity retrieval was more significant for 1/3 LD_{50} ($P < 0.05$) (Figure 3).

Myeloperoxidase activity

Colonic myeloperoxidase (MPO) activity in 1/3 LD_{50} phosalone group was noticeably higher than that of the normal and EA groups ($P < 0.01$). Data showed a remarkable difference between 1/5 LD_{50} phosalone and EA group ($P < 0.01$). Also, the group of animals which received EA and 1/3 LD_{50} phosalone, showed a reduction of MPO activity (by 26%, $P < 0.05$) in comparison to 1/3 LD_{50} phosalone group (Figure 4).

Oxidative-stress as TBARS

Inflammation in colon referred as over-activity of oxidative stress was found high in 1/3 and 1/5 LD_{50} phosalone groups as compared to normal and EA groups ($P < 0.01$). Colonic lipid peroxidation in 1/10 LD_{50} phosalone group was noticeably higher than that of the normal group ($P < 0.01$). Although, EA decreased oxidative stress in all doses of phosalone, it down-regulated oxidant formation significantly in 1/5 LD_{50} phosalone ($P < 0.05$) (Figure 5).

TTM

An obvious reduction in TTM was observed in 1/3 LD_{50} phosalone group as compared to normal and EA groups ($P < 0.01$). 1/5 and 1/10 LD_{50} phosalone groups significantly decreased TTM in comparison with normal group ($P < 0.05$). EA restored significantly the TTM which was suppressed by 1/3 LD_{50} phosalone (Figure 6).

Anti-oxidant power as FRAP

Less ability in overcoming the oxidative stress in all doses of phosalone groups was reported in contrast to normal and EA groups ($P < 0.001$). FRAP value in 1/3 LD_{50} phosalone was significantly less than EA and 1/3 LD_{50} phosalone group ($P < 0.01$). Amount of FRAP in 1/5 LD_{50} phosalone was markedly lower than its normal content in EA and 1/5 LD_{50} phosalone group ($P < 0.001$). The amount of FRAP increased significantly in EA and 1/10 LD_{50} phosalone group compared to 1/10 LD_{50} phosalone group ($P < 0.001$). A significant increase in FRAP was seen in EA and 1/20 LD_{50} phosalone group when compared to 1/20 LD_{50} phosalone ($P < 0.01$) (Figure 7).

TNF-α level

An obvious rise in TNF-α level was observed in (1/3 and 1/5) LD_{50} phosalone groups as compared to normal group ($P < 0.01$). In (1/3 and 1/5) LD_{50} phosalone groups showed a significant increase in TNF-α level in comparison with EA group ($P < 0.05$). A noticeable improve in TNF-α content was seen in EA and 1/3 LD_{50} phosalone group when compared with 1/3 LD_{50} phosalone group ($P < 0.001$). In EA and 1/5 LD_{50} phosalone group as shown in Figure 8, EA prevented more secretion of TNF-α when compared to 1/5 LD_{50} phosalone group ($P < 0.05$) (Figure 8).

IL-6β level

All doses of phosalone groups showed a notable elevation in IL-6β level in comparison to normal group

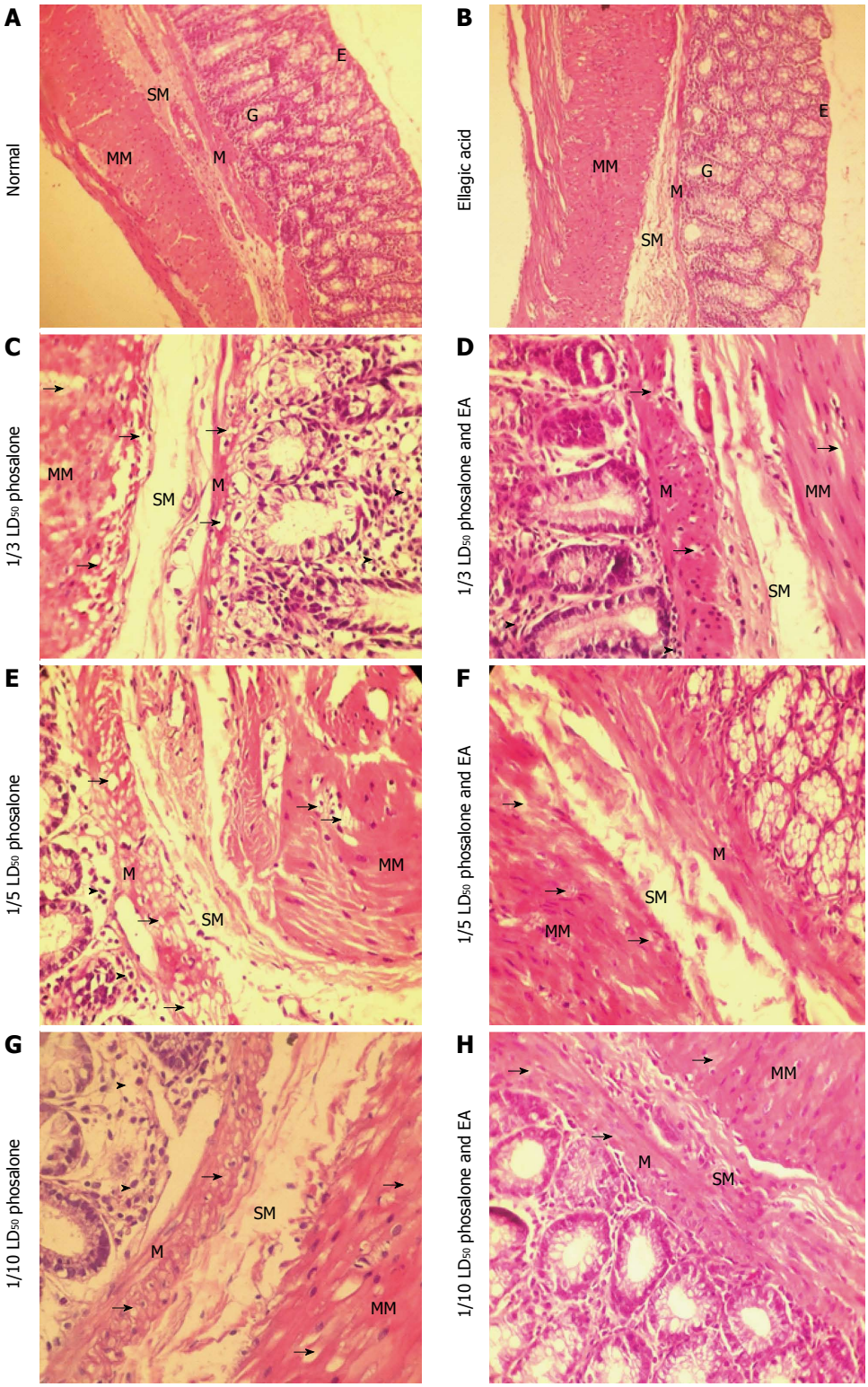
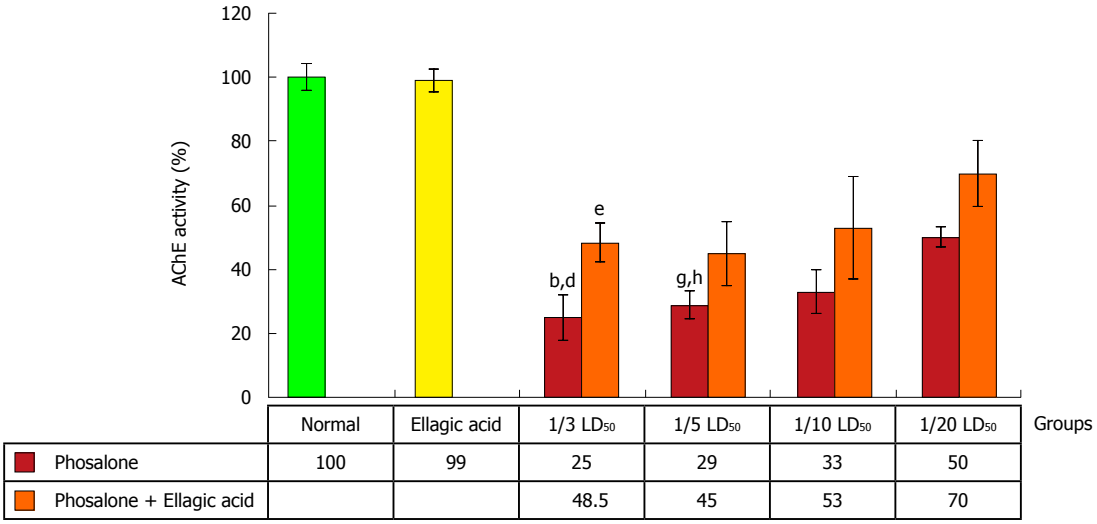


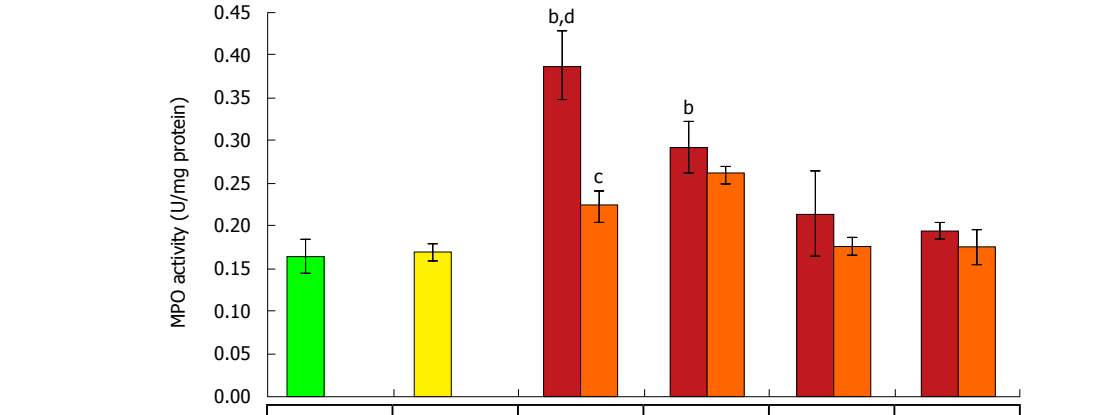
Figure 2 Histological images of colon tissues from normal, ellagic acid and experimental groups. In control group, different parts of the colon tissue are healthy. There are no erosions or ulcers in epithelium. It cannot be seen any necroses and inflammation cells in mucus and mucosal glands in the lamina propria. The mucus thickness, the size of the glands, the muscle layer of mucosal and serous is normal. No degeneration, swelling and goblet cells are observable (A). In ellagic acid (EA) group, the following are normal, the mucosal, the goblet gland cells, epithelium, the mucosa thickness and the size of the glands. There are no ulcers, necrosis hyperplasia and inflammation cells such as neutrophils or lymphocytes. It cannot be observed any degeneration, swelling and goblet cells. The serous is normal without any adherent (B). In 1/3 LD_{50} phosalone group, no necrosis and ulcer are visible in epithelium. The mucosal glands are normal but severe degeneration of mucosal muscle cells and muscle layers is observable. In addition to hyperemia, there is a significant infiltration of mononuclear inflammatory cells such as lymphocytes and plasma cells between the mucosal glands. There is no fibrosis and serous adhesion (C). In 1/3 LD_{50} phosalone group and EA, no necrosis and ulcer are visible in epithelium. The mucosal glands are normal but mild degeneration of mucosal muscle cells and muscle layers is observable. The level of degeneration and inflammation is less than 1/3 LD_{50} phosalone group. There is a very mild inflammation due to lymphocytes infiltration between mucosal glands. There is no serous adhesion (D). In 1/5 LD_{50} phosalone group, no necrosis and ulcer are visible in epithelium. The mucosal glands are normal but relatively severe

degeneration of mucosal muscle cells and muscle layers is observable. In addition to hyperemia, there is a significant infiltration of mononuclear inflammatory cells such as lymphocytes and plasma cells between the mucosal glands. There is no fibrosis and serous adhesion (E). In 1/5 LD_{50} phosalone group and EA, no necrosis and ulcer are visible in epithelium. The mucosal glands are normal but mild degeneration of mucosal muscle cells and muscle layers is observable. The level of degeneration is less than 1/5 LD_{50} phosalone group. There is a very mild inflammation due to lymphocytes infiltration between mucosal glands. There is no serous adhesion (F). In 1/10 LD_{50} phosalone group, no necrosis and ulcer is present in epithelial. The mucosal glands are normal but significant degeneration of muscle cells and mucosal layer is observable. In addition to hyperemia, there is a mild infiltration of mononuclear inflammatory cells such as lymphocytes between the mucosal glands. There is no fibrosis and serous adhesion (G). In 1/10 LD_{50} phosalone group and EA, no necrosis and ulcer are visible in epithelium. The mucosal glands are normal but mild degeneration of mucosal muscle cells and muscle layers is observable. The level of degeneration is less than 1/10 LD_{50} phosalone group. There is a very mild inflammation due to lymphocytes infiltration between mucosal glands. There is no serous adhesion (H). In 1/20 LD_{50} phosalone group, there is no evidence of necroses, ulcer or inflammation in epithelium. Mucosal glands are normal but a mild degeneration is observable in muscle cells in mucosal layer. A mild diapedesis, hyperemia and inflammation in mucosal is visible. There is no fibrosis and serous adhesion (I). In 1/20 LD_{50} phosalone group and EA, no necrosis and ulcer are visible in epithelium. The mucosal glands are normal but mild degeneration of mucosal muscle cells and muscle layers is observable. The level of degeneration is less than 1/20 LD_{50} phosalone group. There is no inflammation in different layers (J). ML: Muscular layer; SM: SubMucosa; M: Mucosa; G: Gland; E: Epithelium.



	Normal	Ellagic acid	1/3 LD_{50}	1/5 LD_{50}	1/10 LD_{50}	1/20 LD_{50}	Groups
Phosalone	100	99	25	29	33	50	
Phosalone + Ellagic acid			48.5	45	53	70	

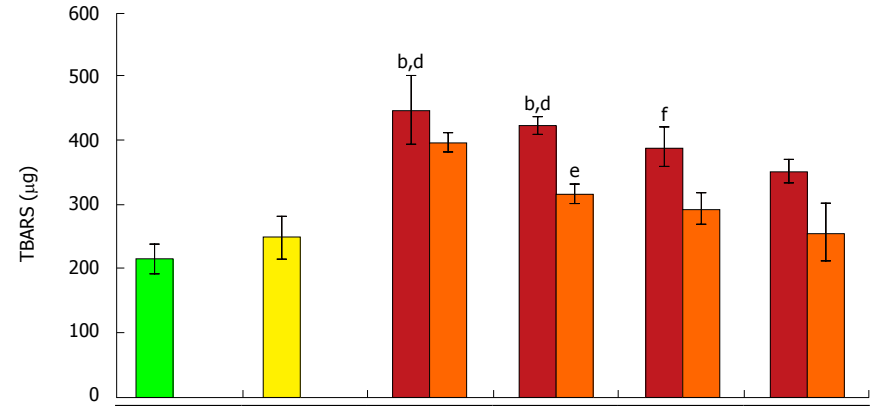
Figure 3 Effect of phosalone and ellagic acid on AChE activity of colon cells. Values are mean ± SE. $^{b}P < 0.001$ *vs* normal group; $^{d}P < 0.001$ *vs* ellagic acid group; Ellagic acid significantly increased of AChE activity in 1/3 dose of phosalone group. $^{e}P < 0.05$ *vs* (1/3 LD_{50} phosalone) group; $^{g}P < 0.05$ *vs* ellagic acid group, $^{h}P < 0.01$ *vs* normal group.



	Normal	Ellagic acid	1/3 LD_{50}	1/5 LD_{50}	1/10 LD_{50}	1/20 LD_{50}	Groups
Phosalone	0.163	0.169	0.387	0.292	0.214	0.194	
Phosalone + Ellagic acid			0.222	0.260	0.175	0.175	

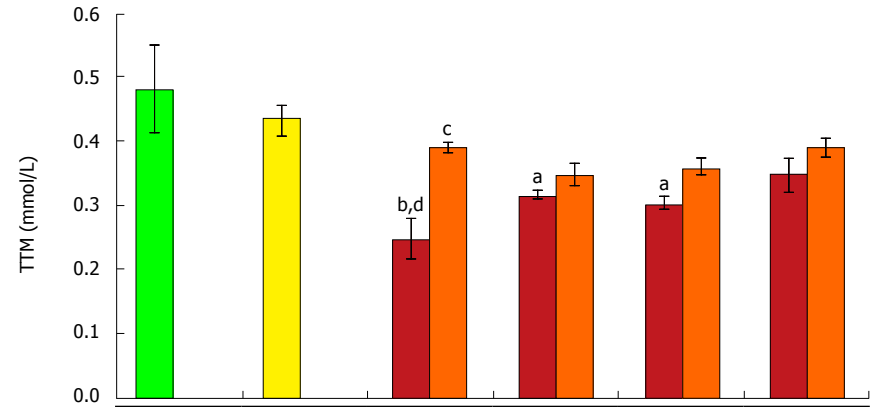
Figure 4 Effect of phosalone and ellagic acid on myeloperoxidase activity of colon cells. Ellagic acid significantly decreased of MPO in 1/3 dose of phosalone group. Values are mean ± SE. $^{b}P < 0.001$ *vs* normal group; $^{c}P < 0.05$ *vs* (1/3 LD_{50} phosalone) group; $^{d}P < 0.01$ *vs* Ellagic acid group. MPO: Myeloperoxidase activity.

($P < 0.001$). The EA and 1/3 dose of phosalone group differed from 1/3 LD_{50} phosalone group remarkably ($P < 0.05$). IL-6β level in 1/3 LD_{50} phosalone group was noticeably higher than that of the EA group ($P < 0.001$). There was significant variation between EA, and EA and 1/5 LD_{50} phosalone groups ($P < 0.01$), while EA and 1/10 LD_{50} phosalone group had a less potency in decreasing IL-6β level when compared to EA group (P



	Normal	Ellagic acid	1/3 LD_{50}	1/5 LD_{50}	1/10 LD_{50}	1/20 LD_{50}	Groups
Phosalone	214.16	247.94	447.46	422.88	390.030	351.88	
Phosalone + Ellagic acid			395.98	317.10	293.516	256.57	

Figure 5 Effect of phosalone and ellagic acid on oxidative-stress as thiobarbituric acid-reaction substances of colon cells. Ellagic acid significantly decreased of thiobarbituric acid-reaction substances in 1/5 dose of phosalone group. Values are mean ± SE. $^{b}P < 0.001$ *vs* normal group; $^{d}P < 0.01$ *vs* Ellagic acid group; $^{e}P < 0.05$ *vs* (1/5 LD_{50} phosalone) group; $^{f}P < 0.01$ *vs* normal group.



	Normal	Ellagic acid	1/3 LD_{50}	1/5 LD_{50}	1/10 LD_{50}	1/20 LD_{50}	Groups
Phosalone	0.482	0.433	0.249	0.317	0.304	0.347	
Phosalone + Ellagic acid			0.391	0.349	0.360	0.391	

Figure 6 Effect of phosalone and ellagic acid on total thiol molecules activity of colon cells. Ellagic acid significantly increased of total thiol molecules in 1/3 dose of phosalone group. Values are mean ± SE. $^{a}P < 0.05$ *vs* normal group; $^{b}P < 0.01$ *vs* normal group; $^{c}P < 0.05$ *vs* (1/3 LD_{50} phosalone) group; $^{d}P < 0.01$ *vs* Ellagic acid group.

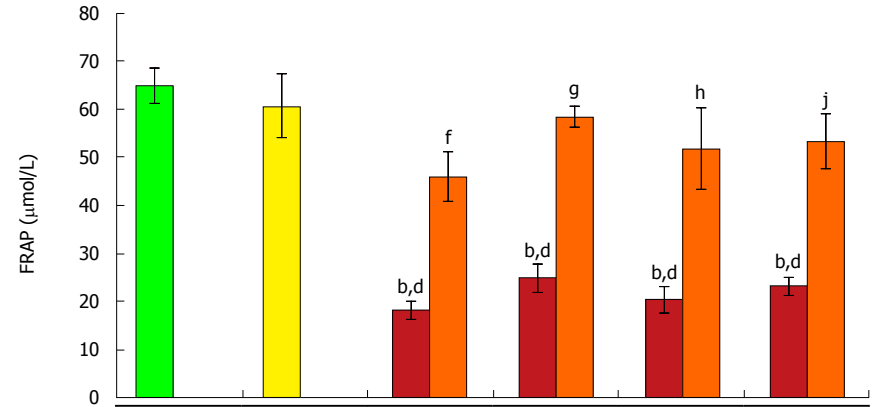
< 0.05) (Figure 9).

NF-κB release

As seen in Figure 10, NF-κB production was significantly elevated in the (1/3 and 1/5) LD_{50} phosalone groups when compared with normal group ($P < 0.001$). A significant increase in NF-κB was seen in (1/10 and 1/20) LD_{50} phosalone groups when compared with normal ($P < 0.05$). The EA and 1/3 LD_{50} phosalone group showed more reduction in NF-κB when compared with 1/3 LD_{50} phosalone ($P < 0.05$). The (1/3, 1/5 and 1/10) LD_{50} phosalone groups which were treated with EA showed an apparent increase in NF-κB level when compared with EA group *(*$P < 0.01$*)*.

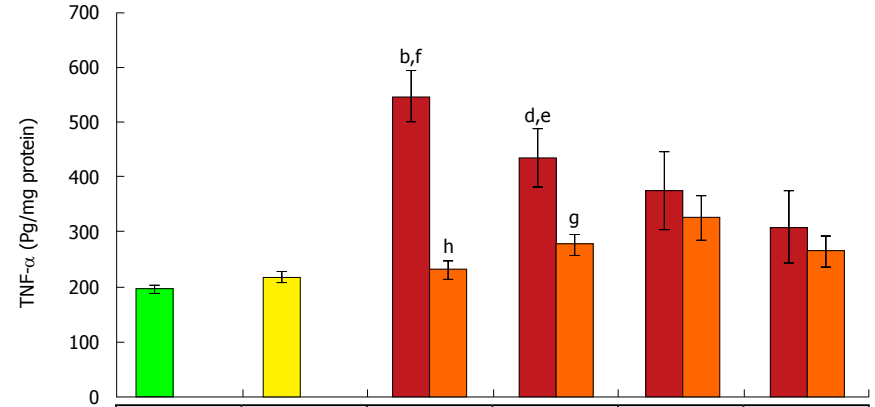
DISCUSSION

In our study we succeeded to achieve our main hypothesis: to find phosalone toxicity in colonic tissues of rats as well as protective effects of EA, during subchronic exposure. Phosalone is type of OP pesticide that could affect different organs in daily and produce various toxicities[31]. As a result of phosalone exposure in rats, increase in oxidative stress and inflammatory markers were observed. In our experiment, EA was used to reduce colon injury induced by phosalone as a protective agent, which showed substantial decrease in oxidative stress and inflammatory markers. EA that is kind of polyphenol derived from different plants



	Normal	Ellagic acid	1/3 LD_{50}	1/5 LD_{50}	1/10 LD_{50}	1/20 LD_{50}	Groups
Phosalone	64.79	60.65	18.22	24.700	20.311	23.25	
Phosalone + Ellagic acid			46.04	58.375	51.790	53.37	

Figure 7 Effect of phosalone and ellagic acid on anti-oxidant power as (ferric reducing antioxidant power) of colon cells. Ellagic acid significantly decreased of ferric reducing antioxidant power in all doses of phosalone groups. Values are mean ± SE. $^{b}P < 0.001$, *vs* normal group; $^{d}P < 0.001$ *vs* ellagic acid group; $^{f}P < 0.01$ *vs* (1/3 LD_{50} phosalone) group; $^{g}P < 0.001$ *vs* 1/5 LD_{50} phosalone; $^{h}P < 0.001$ *vs* 1/10 LD_{50} phosalone; $^{j}P < 0.01$ *vs* 1/20 LD_{50} phosalone.

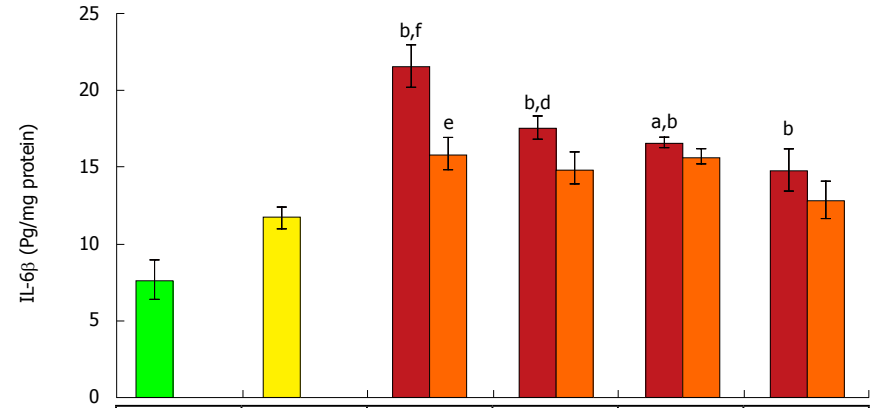


	Normal	Ellagic acid	1/3 LD_{50}	1/5 LD_{50}	1/10 LD_{50}	1/20 LD_{50}	Groups
Phosalone	196	217.9	546.430	433.870	375.125	309.500	
Phosalone + Ellagic acid			230.475	275.355	324.760	264.512	

Figure 8 Effect of phosalone and ellagic acid on tumor necrosis factor-α of colon cells. Ellagic acid significantly decreased of tumor necrosis factor-α in 1/3 and 1/5 doses of phosalone groups. Values are mean ± SE. $^{b}P < 0.001$ *vs* normal group; $^{d}P < 0.01$ *vs* normal group; $^{e}P < 0.05$ *vs* ellagic acid group; $^{f}P < 0.01$ *vs* ellagic acid group; $^{g}P < 0.05$ *vs* (1/5 LD_{50} phosalone) group; $^{h}P < 0.001$ *vs* (1/3 LD_{50} phosalone) group.

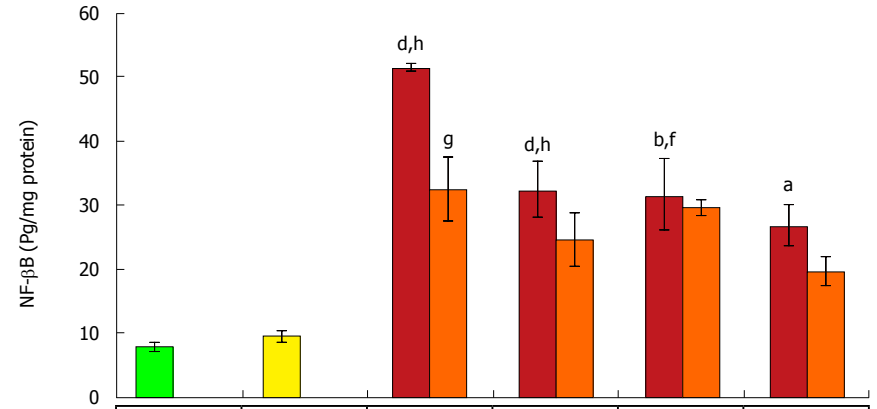
or fruits has already been reported to have sort of protective effects in different diseases[32,33]. As indicated in the present study, AChE activity was reduced with pronounced effect in colon cells of groups receiving (1/3 and 1/5) LD_{50} phosalone in comparison to both normal group and EA group. In previous studies, the same inhibition of AChE was observed during behavioral studies in phosalone-treated rats brain cells[34,35]. AChE inhibition is among best indicator of toxicity induced by any xenobiotic or chemical that initiates other signaling pathways. Despite of little effect in other groups, EA considerably reversed the activity of AChE, suppressed by phosalone in group receiving 1/3 LD_{50} phosalone. It has been already published that, by exposure of OPs elevated level of ACh *via* ChE inhibition could interfere with cholinergic receptors within hypothalamus and potentiate release of adrenocorticotropic hormone (ACTH)[36]. The present study proves that phosalone inhibits AChE activity that is associated with colon inflammation and EA could reverse its effect.

Increased production and decreased ability of ROS and antioxidant defense mechanism respectively can damage various signaling pathways, as well as cell constituents, including DNA, lipids and proteins. Induction of such oxidative impairment *via* redox signaling mechanisms can cause different human diseases[37]. In our experiment, biochemical assays showed that phosalone elevated oxidative stress *via* elevation of MPO activity and TBARS concentration, whereas in groups with combined EA administration; reduction in MPO activity and TBARS concentration has been observed. Irrespective of our study on phosalone,



	Normal	Ellagic acid	1/3 LD_{50}	1/5 LD_{50}	1/10 LD_{50}	1/20 LD_{50}	Groups
Phosalone	7.66	11.72	21.55	17.54	16.60	14.83	
Phosalone + Ellagic acid			15.85	14.91	15.67	12.86	

Figure 9 Effect of phosalone and ellagic acid on interlukin-6β of colon cells. Ellagic acid significantly decreased of tumor necrosis factor-α in 1/3 dose of phosalone group. Values are mean ± SE. $^{a}P < 0.05$ *vs* ellagic acid group; $^{b}P < 0.001$ *vs* normal group; $^{d}P < 0.01$ *vs* ellagic acid group; $^{e}P < 0.05$ *vs* (1/3 LD_{50} phosalone) group; $^{f}P < 0.001$ *vs* ellagic acid group.



	Normal	Ellagic acid	1/3 LD_{50}	1/5 LD_{50}	1/10 LD_{50}	1/20 LD_{50}	Groups
Phosalone	7.78	9.43	51.411	32.36	31.68	26.87	
Phosalone + Ellagic acid			32.470	24.60	29.56	19.65	

Figure 10 Effect of phosalone and ellagic acid on nuclear factor-κB of colon cells. Ellagic acid significantly decreased of nuclear factor-κB in 1/3 dose of phosalone group. Values are mean ± SE. $^{a}P < 0.05$ *vs* normal group; $^{b}P < 0.01$ *vs* normal group; $^{d}P < 0.001$ *vs* normal group; $^{f}P < 0.01$ *vs* ellagic acid group; $^{h}P < 0.001$ *vs* ellagic acid group; Significantly different from at $^{g}P < 0.05$ *vs* (1/3 LD_{50} phosalone) group.

number of previous studies and literature demonstrate a close relation between exposure of pesticides and occurrence of various health problems *via* induction of oxidative stress[38,39]. A study conducted on humans *via in vitro* setup concluded that: oxidative stress and ROS were increased due to OPs exposure[40]. On other hand a pronounced effect of EA against free radical formation can be seen in groups receiving (1/3 and 1/5) LD_{50} phosalone. However, it has been reported that both MPO and TBARS are indicators of oxidative stress and colon inflammation[41,42]. In addition to this, in our study, body's antioxidant defense mechanism was targeted by phosalone, which caused reduction in TTM and FRAP concentration as compared to normal and EA groups. A significant effect of EA as antioxidant has been observed in all groups receiving both EA and phosalone in different doses. Protective and beneficial effects of EA in oxidative stress has been previously evidenced in many studies[43,44]. It can be derived from current biochemical tests that colon tissues of rats are prone to OPs like Phosalone and thus EA can better treat colon tissue damage *via* different mechanisms, while further studies can be conducted for treatment of IBD (inflammatory bowel disease). Oxidative stress and its balance is the most significant feature of normal physiology, in case of high toxicity it can initiate many signaling pathways that can lead to cell death. Our study shows that, EA play its role as protective agent in oxidative impairment, against free radical production and colitis due to phosalone exposure. So in colon inflammation induced by pesticides, EA can be used as anti-inflammatory, anticancer and antioxidant.

Furthermore we evaluated the effect of phosalone on different inflammatory markers along with protective effect of EA. Our concept regarding toxic mechanisms in colon inflammation is growing and general overview is that T cells secrete IL-2, IL-1B and Interferon-c (IFN-c) that can excite macrophages to release extra TNF-α and IFN-c, ROS and other inflammatory mediators. Occurrence of TNF-α, IL-1B, ROS and some antigens target other signaling pathways which ultimately result in synthesis of cytokines[45]. TNF-α employs its action through elevating the synthesis of inflammatory mediators like IL-1 and IL-6[46]. Our recent study shows that phosalone increase TNF-α and EA well treat such condition in colon inflammation, which require further research regarding IBD. Phosalone caused increase in TNF-α level in all groups with significant change in group 1/3 LD_{50} phosalone and 1/5 LD_{50} phosalone as compared to normal and EA groups. EA showed prominent effect as protective agent to reduce TNF-α level in all groups with significant change in group 1/3 LD_{50} phosalone and 1/5 LD_{50} phosalone as compared to others.

In case of biomarker IL-6β, our study showed consistent finding and phosalone caused increase in IL-6β in all groups significantly as compared to normal and EA groups, whereas EA reversed its effect in all groups with significant change in group 1/3 LD_{50} phosalone as compared to EA group. It is common belief that NF-κB shows its significant function in expression of various inflammatory mediators. NF-κB controls transcriptional activity involved in inflammatory and immune process *via* binding to specific DNA sequences in inflammatory genes[47]. In the same pattern, NF-κB was increased in all groups receiving phosalone with significant change. Contrary to this EA reduced its concentration in almost all groups with significant effect of group receiving 1/3 LD_{50} phosalone. In parallel to our current study same effects of EA as anti-inflammatory agent has been observed in previous experiment[48]. It is clear from our results that how EA and phosalone target different biochemical pathways of toxicity. These distinct properties make NF-κB a promising target in novel treatment plans. There are new techniques that directly target NF-κB in inflammatory conditions including antioxidants, antisense DNA targeting, and proteasome inhibitors. Parallel to our study, a previous study also demonstrated that antioxidant effect may also give boost to anti-inflammatory actions[49]. However, EA's mechanism of actions to offset phosalone toxicity can be further studied regarding signaling pathways and gene expressions. Our research outcomes can give new directions, regarding novel treatment plans of colitis as well as awareness of phosalone toxicity in colon tissues.

Our data correlate well with the other studies and demonstrate that phosalone is among one of causative agents to induce colon inflammation and EA is an ideal antioxidant and anti-inflammatory compound in rat modeling studies which has extraordinary effects on oxidant and inflammation systems. Anyhow, additional investigation for *in vivo* and human studies is required. It may indicate a new way toward the development of antioxidant therapy for colon inflammation.

COMMENTS

Background

Pesticides are chemical agents which are used to kill agricultural and domestic insects. Some of the pesticides are based on Organophosphorus (OP) compounds which are also harmful for human and can lead to early aging and cancer. Understanding the mechanism of action of OPs in human body is of prime importance in recent years. Such understanding will lead to finding the means to counteract the side effects resulted from OP exposure. Phosalone is an OP compound used in this study.

Research frontiers

Prior researches have shown that OP exposure causes inflammation and oxidative stress in the body. The previous and on-going research efforts report serious damages to DNA, RNA and cell cycle due to OP agents.

Innovations and breakthroughs

This research confirms the side effects of OP in colon cells in a rat model. Such side effects are the elevated level of inflammation and oxidative stress. The research results shows that among four dosages of phosalone, highest dosage leads to the most significant and serious level of inflammation and oxidative stress. To alleviate such deteriorative side effects, this research proposes utilizing ellagic acid (EA) which is a strong antioxidant. When rats were given EA along with phosalone, the level of inflammation and oxidative stress reduced significantly for the highest dose of phosalone.

Applications

The results of this research can initiate appropriate warnings and precautions to all individuals including farmers who are exposed excessively to OP compounds. Such individuals can be directed to include EA in their diet through taking EA tablets or eating the fruits and vegetable which are rich source of antioxidants and EA like strawberries, grapes and green tea.

Terminology

Reactive oxygen species (ROS) is a physiological process which happens when the body defense system gets triggered due to inflammation and oxidative stress. ROS leads to variety damages to DNA, RNA and cells.

Peer-review

This study is very significant and interesting. The authors have done standard measurements of toxicity, and demonstrated EA can be used to reduce oxidative stress and regulate the level of inflammatory proteins. EA maybe a good candidate which can help treat and alleviate the side effects induced by OP compounds.

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ORIGINAL ARTICLE

Basic Study

CdSe/ZnS quantum dots induce photodynamic effects and cytotoxicity in pancreatic cancer cells

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Data sharing statement: Technical appendix, statistical code, and dataset are available from the corresponding author at leiming.xu@aliyun.com. Participants gave informed consent for data sharing.

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Abstract

AIM: To investigate the photodynamic effect of CdSe/ZnS quantum dots (QDs) on pancreatic cancer cells and elucidate the probable mechanisms.

METHODS: The pancreatic cancer cell line SW1990 was treated with different concentrations of CdSe/ZnS QDs (0, 0.5, 1.0, 1.5, 2.0, 2.5 μmol/L), with or without illumination. The viability of SW1990 cells was tested using the Cell Counting Kit-8 (CCK-8) assay. The ultrastructural changes of SW1990 cells were observed by transmission electron microscopy. Apoptosis was detected by nuclear staining and flow cytometry (FCM). Reactive oxygen species (ROS) were measured

by dichlorofluorescein diacetate *via* fluorescence microscopy. Expression of Bax, Bcl-2 and caspase-3 was measured by real-time polymerase chain reaction (PCR) and protein immunoblotting 24 h after SW1990 cells were treated with CdSe/ZnS QDs and illuminated.

RESULTS: The CCK-8 assay results showed that both CdSe/ZnS QDs with and without illumination suppressed SW1990 cell proliferation. Cell viability was significantly lower when illuminated or with a longer incubation time and a higher light dose. CdSe/ZnS QDs with illumination caused ultrastructural changes in SW1990 cells, such as organelle degeneration and chromatin condensation and aggregation at the periphery of the nucleus. Fluorescence microscopy and FCM showed that CdSe/ZnS QDs (1.5 μmol/L) with illumination increased SW1990 cell apoptosis (53.2%) and ROS generation compared with no illumination. Real-time PCR showed that expression of Bax and caspase-3 was upregulated and Bcl-2 was downregulated. Immunoblotting results were consistent with real-time PCR results. Inhibition of ROS and apoptosis both attenuated QD-photodynamic-therapy-induced cell death.

CONCLUSION: CdSe/ZnS QDs can be used as a photosensitizer to inhibit SW1990 cell proliferation through ROS generation and apoptotic protein expression regulation.

Key words: Quantum dots; Pancreatic cancer; Apoptosis; Photodynamic therapy; Reactive oxygen species

Core tip: This study showed that quantum dots (QDs) may be a potential photosensitizer for photodynamic therapy (PDT) to treat pancreatic cancer by inhibiting SW1990 cell proliferation and inducing apoptosis through reactive oxygen species (ROS) generation. QD-PDT may induce apoptosis through ROS-, caspase-3-mediated apoptotic pathways, with upregulation of apoptosis signaling molecules such as Bax and downregulation of Bcl-2. These findings provide a new application for PDT in pancreatic cancer. However, more preclinical and clinical trials should be undertaken before further clinical application.

He SJ, Cao J, Li YS, Yang JC, Zhou M, Qu CY, Zhang Y, Shen F, Chen Y, Li MM, Xu LM. CdSe/ZnS quantum dots induce photodynamic effects and cytotoxicity in pancreatic cancer cells. *World J Gastroenterol* 2016; 22(21): 5012-5022 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i21/5012.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i21.5012

INTRODUCTION

Pancreatic cancer is a malignant neoplasm with a very poor prognosis. The 5-year survival is < 5% and medium survival is about 6 mo[1]. Surgical resection is the first-choice treatment for pancreatic cancer, however, 80% of patients may already have locally advanced or metastatic cancer when diagnosed, and only 10%-15% are eligible for surgery[2]. The majority of pancreatic cancer patients have to undergo radiotherapy or chemotherapy, although the survival rates of these nonsurgical patients are similar. Thus, there is an urgent need to identify a novel effective therapy.

Photodynamic therapy (PDT) is an innovative method that utilizes a photosensitizing agent or photosensitizer (PS) followed by light exposure to treat various diseases. Reactive oxygen species (ROS) are generated when PSs are activated by illumination and subsequently destroy cancer cells[3]. In PDT, only cells in contact with the PS, light and oxygen are affected, thus PDT is more selective than conventional chemotherapy and radiotherapy[4]. Most PSs are based on a tetrapyrrole structure. The first PS used clinically for cancer therapy was hematoporphyrin derivative, a water-soluble mixture of porphyrins. As hematoporphyrin derivative is purified from porfimer sodium, it has some disadvantages, such as instability in aqueous solution, long-lasting skin photosensitivity, and weak absorption at the therapeutic wavelength of 630 nm[5]. 5-aminolevulinic acid is a second-generation PS. It is a biosynthetic precursor of protoporphyrin IX that needs to be converted to protoporphyrin as an active PS[6]. However, its skin photosensitivity is still an unresolved problem[7]. Therefore, it is necessary to develop new PSs to confer survival benefits with fewer side effects.

Quantum dots (QDs) are colloidal semiconductors and mainly composed of group Ⅱ-Ⅵ or group Ⅲ-Ⅴ elements[8]. QDs are of interest to many researchers due to their unique optical properties. QDs possess several characteristics such as large absorption spectra, narrow and symmetric emission bands, and a high molar extinction coefficient, which make them superior to conventional PSs in PDT[9,10]. Recently, many studies have shown the potential applications of QDs for PDT. With illumination, the QD conduction-band electron can be transferred to surrounding O_2 and produce ROS, thus making QDs a potential PS for PDT[11,12].

In this study, we prepared water-dispersible CdSe/ZnS QDs with an extensive absorption in the UV-visible region and a strong emission peaking at 560 nm. We investigated the photodynamic effects of CdSe/ZnS QDs on pancreatic cancer cells, and analyzed the possible molecular mechanism involved in this procedure.

MATERIALS AND METHODS

QD nanocrystal characterization

Nanocrystals with a CdSe core and ZnS shell were synthesized by Professor Zhang *et al* at the Department of Materials Science and Engineering, Shanghai

University. Trioctylphosphine oxide, CdO and tetradecylphosphonic acid were heated to 180 ℃ under argon, exsiccated and exhausted under a vacuum. When the reaction temperature reached 330 ℃, selenium precursor solution was added to trioctylphosphine and mixed together until the temperature decreased to 240 ℃. ZnS stock solution was added along with dimethyl zinc solution and vigorously stirred until the molar ratio of Cd/Se: Zn/S reached 1:4. The mixture was cooled to room temperature and settled with anhydrous methanol, centrifuged (4000 rpm) and washed three times with anhydrous methanol to remove residua such as trioctylphosphine oxide and unreacted reagents. The precipitate was suspended in phosphate-buffered saline (PBS). The morphology of QDs was observed using a Morgagni 268(D) transmission electron microscope (FEI, Hillsboro, OR, United States). The UV-Vis spectra of the QDs suspensions were scanned within the wavelength range of 200-800 nm at 22 ℃ and automatically corrected for the suspension, using an Avantes UV-Vis spectrophotometer (Apeldoorn, The Netherlands).

Cell culture

The human pancreatic cancer cell line SW1990 was purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The SW1990 cells were cultured in RPMI 1640 media with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin, in humidified air containing 5% CO_2 at 37 ℃. Cells in the exponential growth phase were used in the following experiments.

Cytotoxicity assays

The cytotoxicity induced by QDs and PDT was determined using the Cell Counting Kit-8 (CCK-8) assay. The cells were seeded on a 96-well plate at 8×10^3 cells/well and incubated overnight before QDs were added. The cells were divided into two groups (A with illumination and B without illumination). The cells in each group were treated with different concentrations of QDs (0, 0.5, 1.0, 1.5, 2.0, 2.5 μmol/L) for 1 h. After incubation, all cells were washed with PBS to remove excess QDs and fresh media were added. Cells in Group A were irradiated using ZF-20D Ultraviolet Analyzing Equipment at a wavelength of 365 nm and power of 19 mW cm^{-2}, and then incubated in RPMI 1640 medium with 10% FBS for a further 24 h at 37 ℃ in a humidified 5% CO_2 atmosphere. However, the medium in Group B was removed and replaced with RPMI 1640 medium with 10% FBS, and then the cells were incubated in humidified air containing 5% CO_2 at 37 ℃ for a further 24 h. After 24 h incubation, CCK-8 dye (Dojindo Laboratories, Kamimashiki-gun, Kumamoto, Japan) was added to each well, and the 96-well plates were put into a constant temperature incubator (37 ℃) for 1 h. The absorbance of the solution was measured at 450 nm using an ELISA reader (Thermo Fisher Scientific, MA, United States). Cell viability was calculated as a percentage of the treated samples relative to untreated controls.

Subcellular damage of QDs using transmission electron microscopy

Transmission electron microscopy (TEM) was used to investigate the intracellular localization and subcellular structural targets of QDs in SW1990 cells. The cells were seeded on six-well plates at 4×10^5 cells/well and cultured overnight. Twenty-four hours later, after treatment [A: normal SW1990 cells; B: CdSe/ZnS QDs (1.5 μmol/L, 3 h) and illumination; C: CdSe/ZnS QDs (2 μmol/L, 3 h) and illumination (20 J/cm^2)], the cells were collected and washed three times with cold PBS, pelleted using centrifugation (1000 rpm), and fixed in 2.5% glutaraldehyde for 2 h. Cell pellets were washed in PBS, postfixed with 1% osmium tetroxide, and dehydrated with an ascending series of alcohols. The specimens were cut into ultrathin sections (50-70 nm), placed onto copper grids, and stained with uranyl acetate and lead citrate for ultrastructural analysis using a JEM-1011EX transmission electron microscope (Jeol, Tokyo, Japan).

Apoptosis by flow cytometry

The Annexin V-FITC Apoptosis Detection Kit (BD Pharmingen, San Jose, CA, United States) was used to detect QD-induced apoptosis of SW1990 cells. Cells (2×10^5) were seeded in six-well plates and allowed to adhere overnight. The cells were treated [A: normal SW1990 cells; B: SW1990 cells with illumination (20 J/cm^2); C: SW1990 cells treated with CdSe/ZnS QDs (1.5 μmol/L, 3 h); D: SW1990 cells treated with CdSe/ZnS QDs (1.5 μmol/L, 3 h) with illumination (20 J/cm^2)], collected and washed twice with cold PBS. The cell pellets were resuspended in binding buffer, and incubated with staining solution [annexin V/propidium iodide (PI) = 1:1] in the dark for 15 min at room temperature. Fluorescence-activated cell sorting (FACS) analysis was performed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, United States).

Measurement of ROS generation

SW1990 cells were seeded on six-well plates at 4×10^5 cells/well and exposed to QDs as for flow cytometry (FCM) after the cells adhered. After 24 h, the cells were rinsed with cold PBS and stained with 2′,7′-dichlorofluorescin diacetate (H_2DCFDA; Sigma-Aldrich, St. Louis, MO, United States) diluted in serum-free medium. After incubation for 30 min at 37 ℃ in the dark, the cells were washed with serum-free medium three times and resuspended in cold PBS. The DCF fluorescence was observed by a fluorescence microscope (BD Biosciences) and the fluorescence intensity was measured by FCM (BD Biosciences, San Jose, CA, United States). To investigate the role of

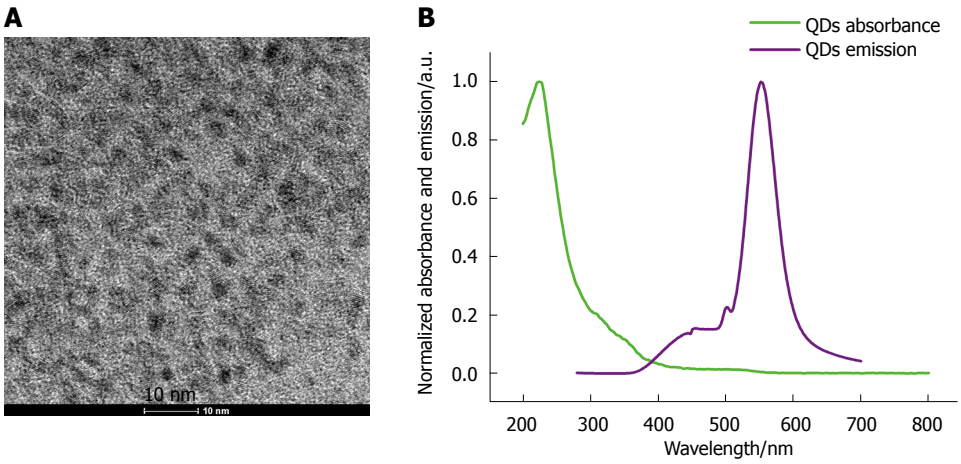


Figure 1 Characterization of CdSe/ZnS quantum dots. A: TEM image of CdSe/ZnS QDs; B: Absorbance and emission of CdSe/ZnS QDs. QDs: Quantum dots.

ROS production in the cytotoxicity of QD-PDT, SW1990 cells were preincubated with 5 mmol/L N-acetylcysteine (NAC) (Sigma-Aldrich), a ROS scavenger, for 1 h before treatment. Cell viability was evaluated by the CCK-8 assay.

Transcriptional analysis of time course in response to QD-PDT

Real-time RT-PCR was performed using 7500/7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, United States) to analyze the apoptosis-related mRNA expression of QD-PDT treated SW1990 cells, such as Bax, Bcl-2 and caspase-3. The primers used in the real-time RT-PCR assay were Bax forward (5′-GGAAGAAGATGGGCTGAGG-3′); Bax reverse (5′-TGTCCCGAAGGAGGTTTATT-3′); Bcl-2 forward (5′-CCGGATCACCATCTGAAGAG-3′); Bcl-2 reverse (5′-AGGGCAAAGAAATGCAAGTG-3′); Caspase-3 forward (5′-AGATGGTTTGAGCCTGAGCA-3′); Caspase-3 reverse (5′-CAGTGCGTATGGAGAAATGG-3′); and GAPDH forward (5′-TGCACCACCAACTGCTTAG-3′); GAPDH reverse (5′-GGATGCAGGGATGATGTTC-3′).

Immunoblotting

Proteins were resolved by SDS-PAGE and blotted onto polyvinylidene difluoride membranes. Anti-Bcl-2 (dilution 1:1000; Cell Signaling Technology, Danvers, MA, United States;), anti-Bax (dilution 1:1000; Cell Signaling Technology), anti-caspase 3 (dilution 1:1000; Cell Signaling Technology), anti-cleaved caspase-3 (dilution 1:1000; Cell Signaling Technology) and anti-β-actin (dilution 1:1000; Cell Signaling Technology) antibodies were used to detect their corresponding proteins followed by anti-rabbit or anti-mouse IgG secondary antibodies (dilution 1:1000; Cell Signaling Technology). Image acquisition was performed with the ChemiDoc XRS+ system (Bio-Rad, Hercules, CA, United States). The optical densities of the protein bands were measured by GS710 Densitometer and analyzed with Quantity One image analysis software (Bio-Rad Laboratories).

Statistical analysis

All experiments were performed in triplicate. The results were expressed as mean ± SD and analyzed by the Student's *t* test with SPSS version 13.0 (SPSS Inc., Chicago, IL, United States). Comparisons among multiple groups of data were analyzed by one-way analysis of variance. $P < 0.05$ was considered statistically significant.

RESULTS

Synthesis and characterization of QDs

QDs were synthesized as previously described. The TEM results showed that QDs were spherical particles with an average size of 5 nm. The peaks of QDs in the UV-Vis analysis showed that the absorbance of QDs was the highest in the UV part of the spectrum, and decreased exponentially when approaching higher wavelengths. The photoluminescence spectra demonstrated that QDs have highest luminescence in the visible part of the spectrum, especially at 560 nm (Figure 1).

Cytotoxicity of QDs

The CCK-8 assay was used to examine the viability of SW1990 cells after different treatments. Cell viability was decreased when the concentration of QDs increased (Figure 2A). Longer incubation time led to lower viability (Figure 2B). Cell viability showed a greater reduction with illumination (Figure 2C). QDs with illumination induced more cytotoxicity in SW1990 cells than QDs alone. More cell damage occurred when the light dose was higher. Illumination alone (10, 20 and 30 J/cm^2) without QDs had limited effects on SW1990 cells (Figure 2C).

The QD-PDT-induced subcellular damage of SW1990 cells was detected by TEM (Figure 3). Under normal conditions, SW1990 cells had a round shape

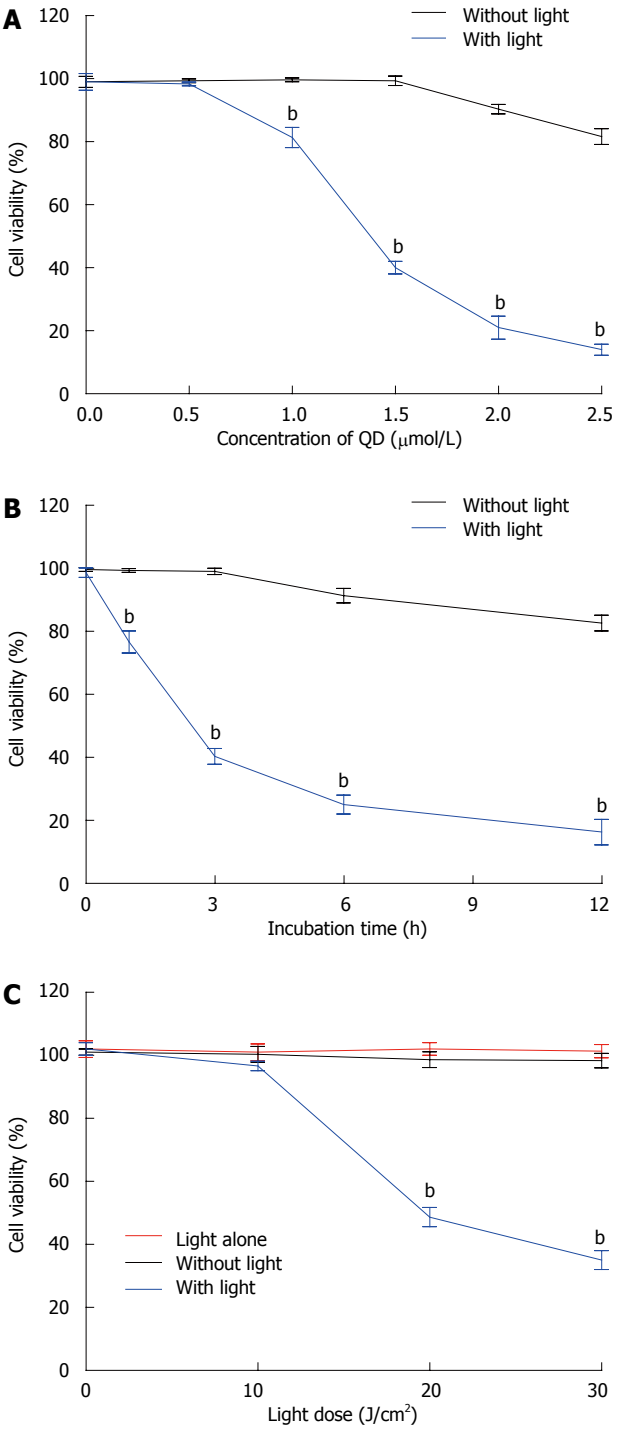


Figure 2 Cell viability of SW1990 cells was inhibited by CdSe/ZnS quantum dots with or without illumination. A: SW1990 cells were treated with various concentrations of CdSe/ZnS QDs (0, 0.5, 1.0, 1.5, 2.0, 2.5 μmol/L); incubation time was 3 h; light dose was 20 J/cm^2; B: SW1990 cells incubated with CdSe/ZnS QDs (1.5 μmol/L) for 0, 1, 3, 6 and 12 h and illuminated (light dose was 20 J/cm^2); C: SW1990 cells alone or incubated with CdSe/ZnS QDs (1.5 μmol/L) for 3 h and illuminated with different light doses (0, 10, 20 and 30 J/cm^2). $^bP < 0.01$ *vs* related group without light. QDs: Quantum dots.

and well-structured mitochondria in the cytoplasm. The cell nucleus was round or class round in the middle of cytoplasm. Nevertheless, after treatment with QDs and illumination, SW1990 cells were significantly

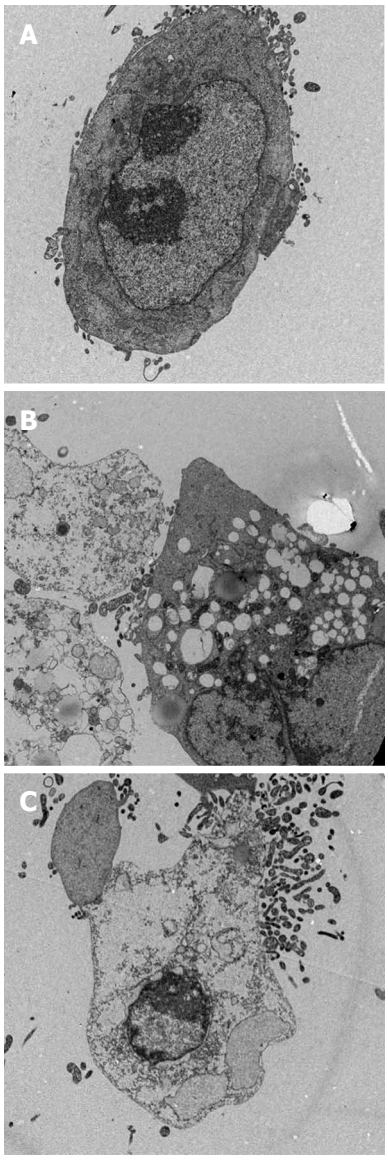


Figure 3 Ultrastructural changes in SW1990 cells induced by CdSe/ZnS quantum dots with or without illumination (TEM, magnification × 2000). A: normal SW1990 cells; B: treated with CdSe/ZnS QDs (1.5 μmol/L, 3 h) and illumination (20 J/cm^2); C: treated with CdSe/ZnS QDs (2 μmol/L, 3 h) and illumination (20 J/cm^2). QDs: Quantum dots.

damaged. Vacuoles and irregularly sized mitochondria appeared. Organelle degeneration, and chromatin condensation and aggregation at the periphery of the nucleus were observed (Figure 3B and C). The main difference between treatment with 1.5 and 2 μmol/L was the percentage of apoptotic and dead cells, thus the latter induced more cell death.

The percentage of apoptotic and necrotic cells was analyzed by fluorescence microscopy and FCM. SW1990 cells were stained with PI and Hoechst 33342. There were more apoptotic bodies in Group D and several cells were even stained with red fluorescence (Figure 4). FCM indicated that the percentage of apoptotic cells was higher in Group D, however, the percentage of necrotic cells remained at a low level

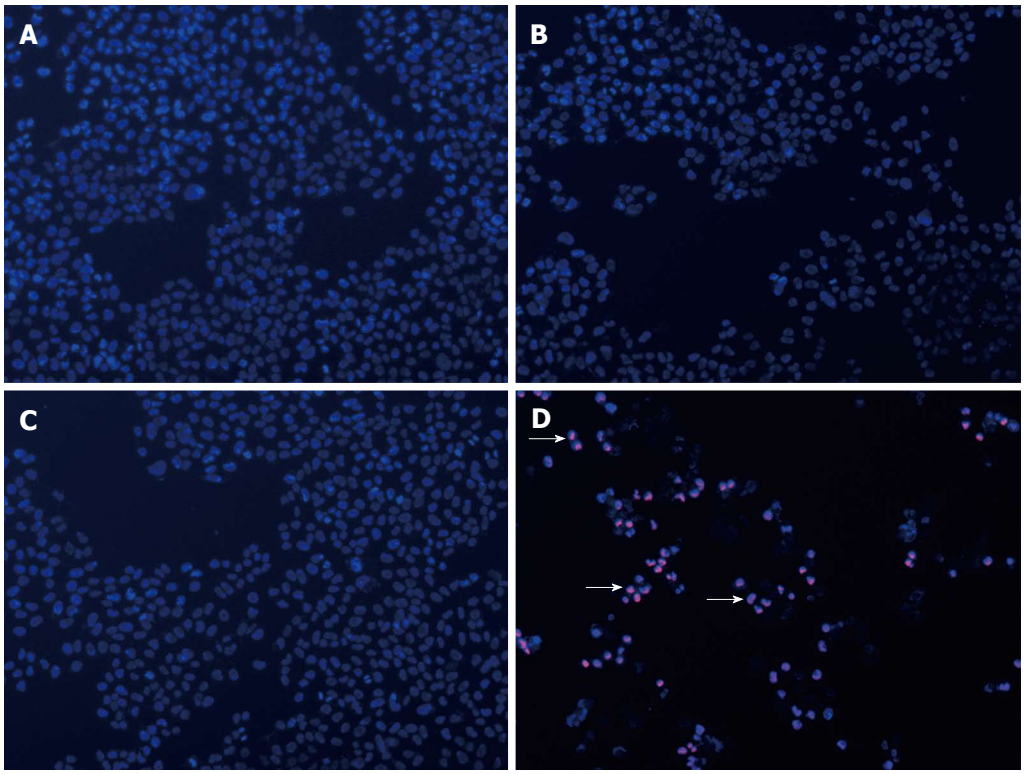


Figure 4 Apoptosis and necrosis were observed in SW1990 cells treated by CdSe/ZnS quantum dots with illumination (Hochest 33342/PI nucleus staining, magnification × 100). A: normal SW1990 cells; B: SW1990 cells with illumination (20 J/cm^2); C: SW1990 cells treated with CdSe/ZnS QDs (1.5 μmol/L, 3 h); D: SW1990 cells treated with CdSe/ZnS QDs (1.5 μmol/L, 3 h) and illumination (20 J/cm^2). The white arrow shows dead cells. QDs: Quantum dots.

(Figure 5).

Measurement of ROS generation

ROS generation was determined by DCF fluorescence in SW1990 cells. The intracellular ROS content in SW1990 cells treated with light (20 J/cm^2) was increased. However, ROS formation significantly increased in SW1990 cells with QDs (1.5 μmol/L, 3 h) and PDT (1.5 μmol/L, 3 h, 20 J/cm^2), especially in cells with PDT (Figure 6A and B). These results indicated that illumination enhanced ROS generation in SW1990 cells treated with QDs.

Expression of mRNA and protein

The mRNA expression level of Bax, Bcl-2 and caspase-3 was measured by RT-PCR. The expression level of each gene was normalized to GAPDH. The mRNA expression level of Bax and caspase-3 increased significantly as compared to control cells, while the level of Bcl-2 decreased (Figures 7 and 8). The protein expression level of these three genes was consistent with corresponding mRNA expression.

Effects of ROS and caspase inhibitors on QD-induced PDT

Pretreatment with an antioxidant (NAC), markedly restored cell viability of SW1990 cells after QD-PDT treatment (Figure 6C), which verified the role of ROS in QD-PDT-induced cytotoxicity. To demonstrate the role of apoptosis in QD-PDT, the pan-caspase inhibitor Z-VAD-FMK was added to the cell culture 1 h before treatment. Inhibition of caspase activation by Z-VAD-FMK abrogated QD-PDT-induced cell death (Figure 9).

DISCUSSION

PDT has been widely used clinically to treat a wide range of malignant cancers, such as esophageal and skin cancer. PDT consisted of two parts: administration of a PS and exposure to light to activate the agent[13,14]. In this study, we synthesized QDs with a CdSe core and ZnS shell and demonstrated the possible QD-induced PDT effects on pancreatic cancer cells.

Selection of an appropriate light wavelength was important. Blue light resulted in inefficient tissue penetration, unlike red and infrared radiation. The range of 600-1200 nm was considered the optical window for tissue penetration. However, only light < 800 nm could generate $^1O_2$, and light > 800 nm could not provide sufficient energy to initiate photosensitization[15]. Thus, there is no ideal single light source for all PDT reactions, even with the same PS. In this study, we selected 365 nm as our illumination wavelength, which happened to be the appropriate excitation wavelength for CdSe/ZnS QDs.

It is reported that cadmium induces ROS generation and triggers apoptosis *via* a caspase-dependent pathway[16,17]. Recently, some studies have shown that

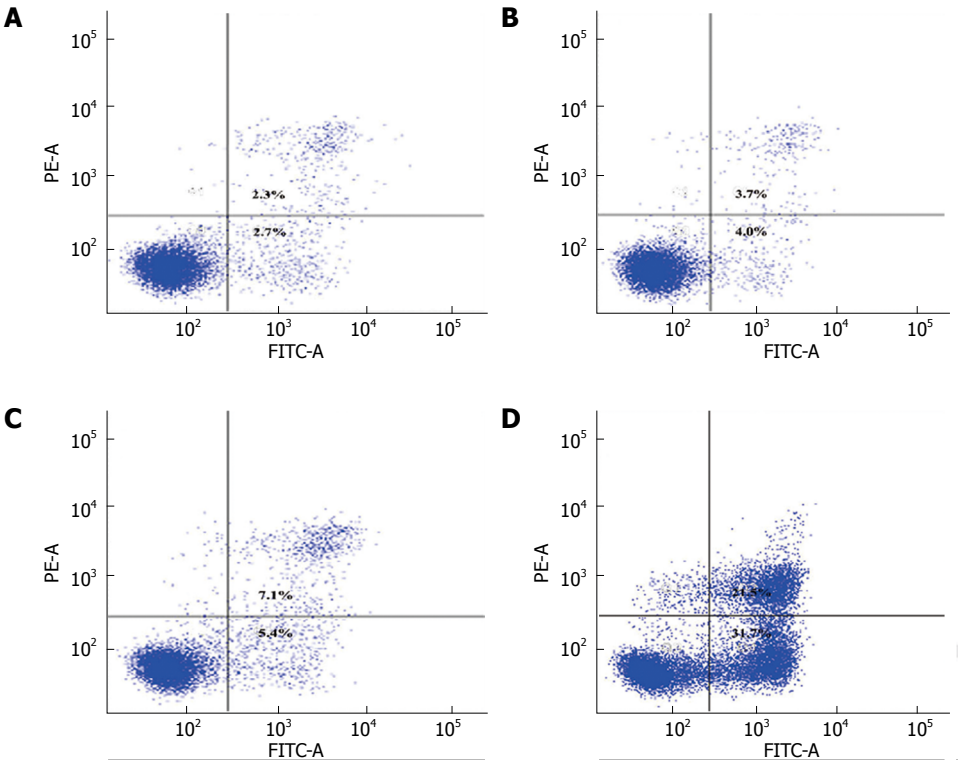
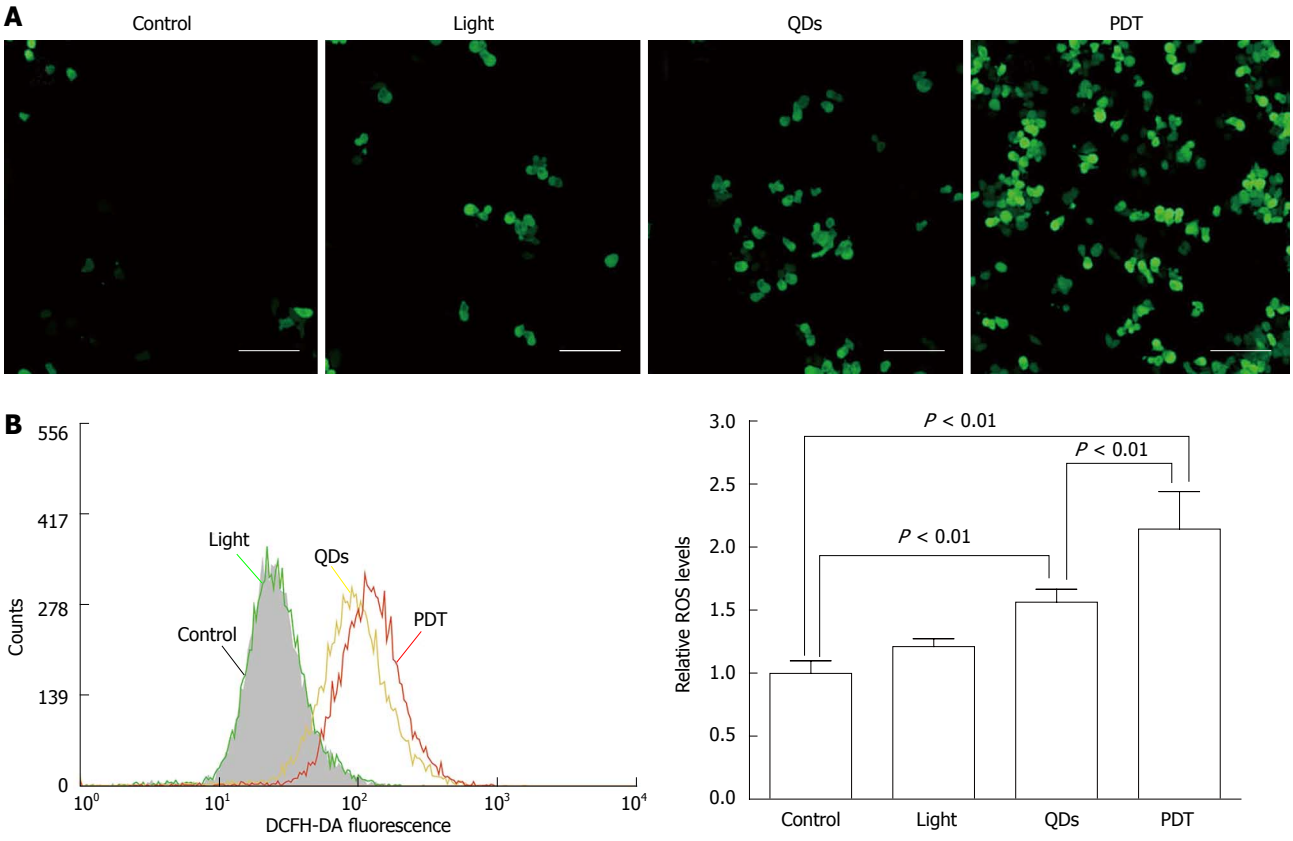


Figure 5 Apoptosis and necrosis were observed in SW1990 cells treated by CdSe/ZnS quantum dots with illumination. A: normal SW1990 cells; B: SW1990 cells with illumination (20 J/cm^2); C: SW1990 cells treated with CdSe/ZnS QDs (1.5 μmol/L, 3 h); D: SW1990 cells treated with CdSe/ZnS QDs (1.5 μmol/L, 3 h) and illumination (20 J/cm^2). FCM of SW1990 cells showed that SW1990 cells had a higher apoptosis rate (53.2%) in Group D than in Groups A, B and C. QDs: Quantum dots.



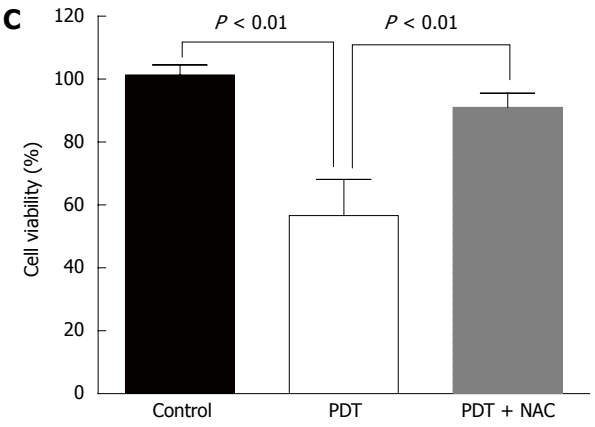


Figure 6 Reactive oxygen species generation was detected after treatment of CdSe/ZnS quantum dots with illumination. A: Fluorescent images of ROS in SW1990 cells (Bar: 200 μm); B: Relative ROS level measured by FCM; C: Cell viability of SW1990 cells by CCK-8 assay. Control: normal SW1990 cells; Light: SW1990 cells with illumination (20 J/cm^2); QDs: SW1990 cells treated with CdSe/ZnS QDs (1.5 μmol/L, 3 h); PDT: SW1990 cells treated with CdSe/ZnS QDs (1.5 μmol/L, 3 h) and illumination (20 J/cm^2). NAC: N-acetylcysteine, a ROS scavenger, 5 mmol/L NAC was added to the cell culture for 1 h before treatment. QDs: Quantum dots; ROS: Reactive oxygen species.

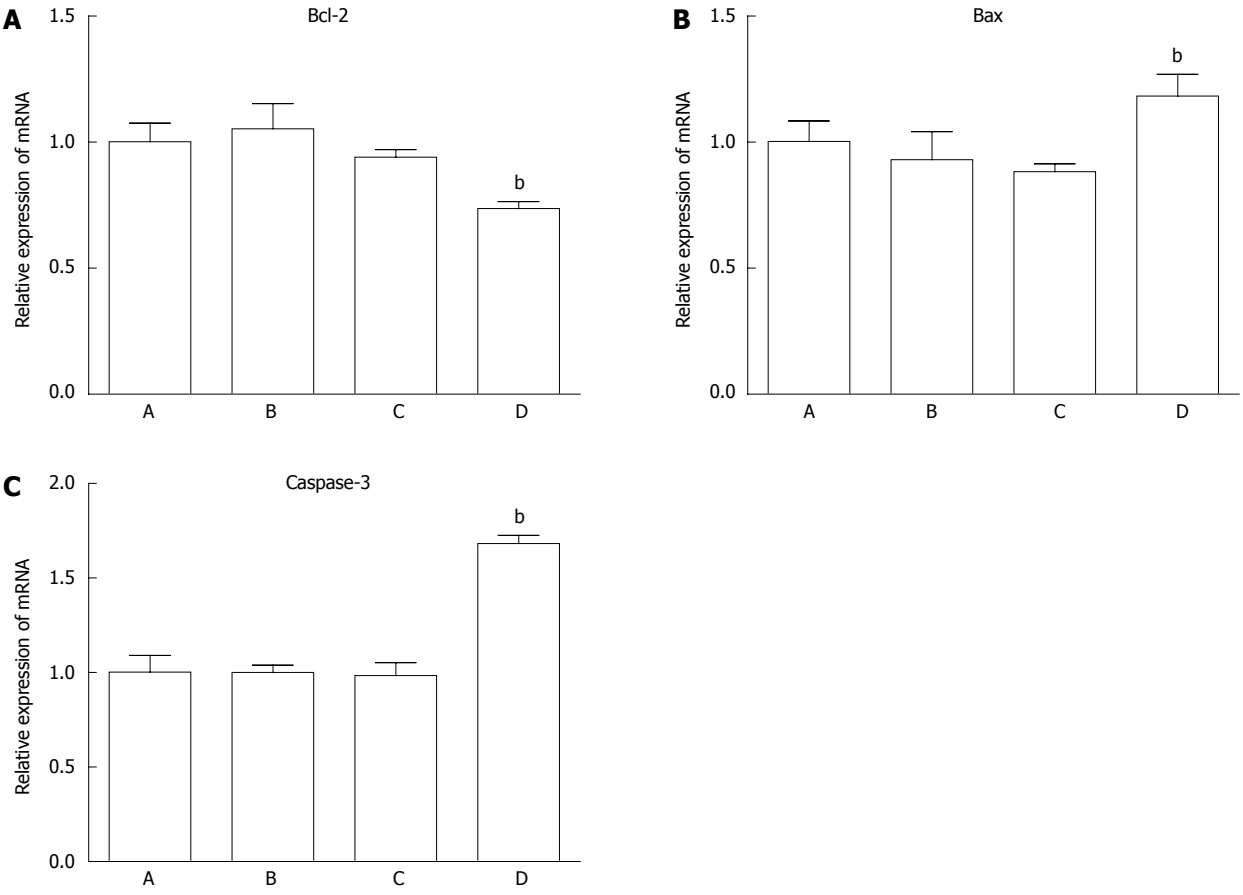


Figure 7 Changes in mRNA expression levels of Bax, Bcl-2 and caspase-3 following CdSe/ZnS quantum dots with illumination. A: Normal SW1990 cells; B: SW1990 cells with illumination (20 J/cm^2); C: SW1990 cells treated with CdSe/ZnS QDs (1.5 μmol/L, 3 h); D: SW1990 cells treated with CdSe/ZnS QDs (1.5 μmol/L, 3 h) and illumination (20 J/cm^2). $^bP < 0.01$ *vs* group A. QDs: Quantum dots.

CdSe-core QDs induce cell death by releasing free Cd^{2+} from the CdSe lattice, and this effect could be impeded by the addition of a coating such as ZnS[18]. Here, we synthesized water-soluble CdSe/ZnS QDs. A ZnS coating made the QDs more biocompatible with cells. However, it effectively reduced ROS generation. QDs are generally used as bioimaging probes for tracing and immunostaining cells[19,20]. In this study, QDs were used as photosensitizers in PDT of the pancreatic cancer cell line SW1990. QDs with ZnS coating showed less cytotoxicity in the dark, even when incubated with cells for 12 h at a concentration of 1.5 μmol/L.

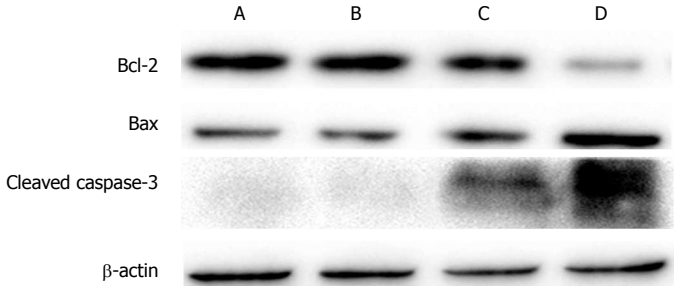


Figure 8 Changes in protein expression levels of Bax, Bcl-2 and caspase-3 following CdSe/ZnS quantum dots with illumination. A: Normal SW1990 cells; B: SW1990 cells with illumination (20 J/cm^2); C: SW1990 cells treated with CdSe/ZnS QDs (1.5 μmol/L, 3 h); D: SW1990 cells treated with CdSe/ZnS QDs (1.5 μmol/L, 3 h) and illumination (20J/cm^2). QDs: Quantum dots.

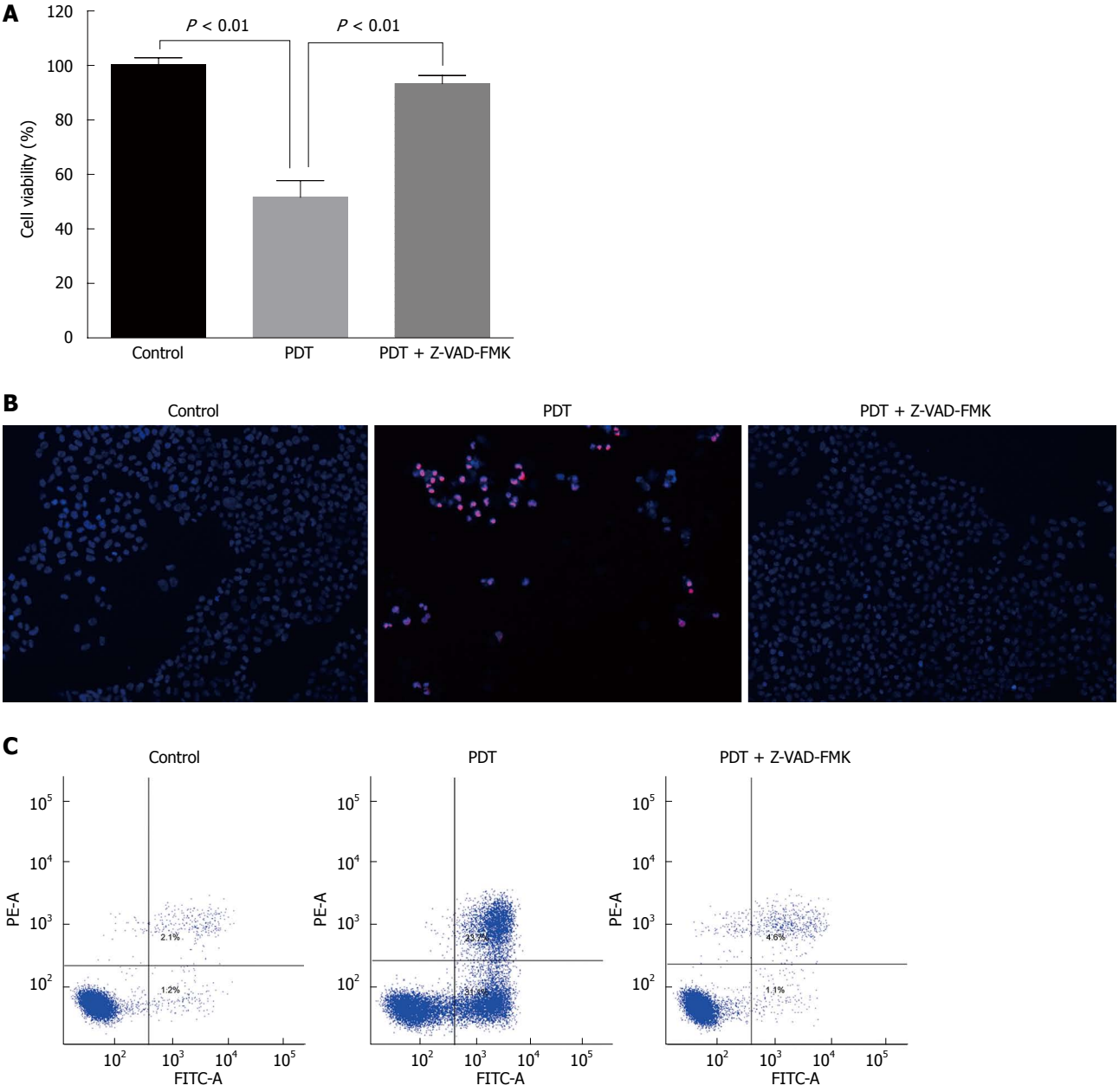


Figure 9 Quantum dots-photodynamic therapy-induced cell death *via* apoptosis. A: Cell viability of SW1990 cells by CCK-8 assay; B: Hochest 33342/PI nucleus staining of apoptotic and necrotic SW1990 cells (magnification × 100), white arrow indicates dead cells; C: Percentage of apoptotic and necrotic SW1990 cells by FCM. SW1990 cells were pretreated with or without Z-VAD-FMK (50 μmol/L) for 1 h and then treated with QD-PDT (1.5 μmol/L, 3 h, 20 J/cm^2). QDs: Quantum dots; PDT: Photodynamic therapy; FCM: Flow cytometry; CCK-8: Cell Counting Kit-8; PI: Propidium iodide.

However, when irradiated by UV, the cytotoxicity of QDs was apparent. Cell viability was decreased when the concentration of QDs increased and incubation time with QDs and light dose increased, which was similar to other studies[21]. TEM, fluorescence microscopy, and FCM illustrated the ability of QDs to generate PDT.

During PDT, ROS generation increased, as reported by Waterhouse *et al*[22], who suggested that the mitochondrion was a vital organelle in programmed cell death and could be mediated by many regulatory factors of apoptosis. To determine whether ROS were increased in QD-induced PDT, we used a probe to detect intracellular ROS variation. Surprisingly, even when coated with ZnS, QDs still generated ROS after illumination, which was statistically significant compared with the control, light and QDs groups. Inhibition of ROS generation with NAC attenuated the cytotoxicity of QD-induced PDT of pancreatic cancer cells, thus ROS were important in this procedure.

To investigate the molecular mechanism of QD-induced PDT, we chose three representative proteins (Bcl-2, Bax and caspase-3) to identify their connection with QD-induced PDT. In this study, we observed apoptosis during QD-induced PDT. Apoptosis has been widely studied and is believed to be triggered by several signals, including a series of proteins[23,24]. The Bcl-2 family of proteins constitutes a central checkpoint[25]. Bax and Bcl-2 are two members of the Bcl-2 family and function as regulatory proteins[26,27]. In this study, we found that QDs increased Bax expression and decreased Bcl-2 expression at the mRNA and protein levels. Several studies have clearly defined Bax as a proapoptotic protein and Bcl-2 as an antiapoptotic protein[28]. In our study, Group D (cells with PDT) showed higher expression of Bax and lower expression of Bcl-2, which to some degree explained the greater apoptosis and necrosis in this group. These results were consistent with other studies[29,30]. Caspase-3 is a member of the cysteine-aspartic acid protease family. As an executioner, caspase-3 is practically inactive until it is cleaved by an initiator caspase when apoptotic signaling events occur. Caspase-3 can be activated in apoptotic cells through extrinsic or intrinsic pathways[31-34]. In this study, cleaved caspase-3 was observed after cells were treated with QDs and illumination. To confirm that apoptosis was involved in the QD-induced PDT effects on pancreatic cancer cells, Z-VAD-FMK was used to restore cell survival and indeed promote cell survival. These results indicated that Bcl-2, Bax and caspase-3 participated in the process of QD-PDT-induced apoptosis. Specifically, QD-PDT downregulated Bcl-2, upregulated Bax, and facilitated caspase-3 cleavage, thus promoting the killing of pancreatic cancer cells.

In summary, this study showed that QDs could be potential PSs for PDT to treat pancreatic cancer by inhibiting SW1990 cell proliferation and inducing apoptosis through ROS generation. QD-PDT may induce apoptosis through ROS-, caspase-3-mediated apoptotic pathways, with upregulation of apoptosis signaling molecules such as Bax and downregulation of Bcl-2. These findings provide a new application for PDT in pancreatic cancer. However, more preclinical and clinical trials should be undertaken before further clinical application.

COMMENTS

Background

Pancreatic cancer is one of the most malignant tumors and has a poor prognosis. Conventional treatments such as surgery, chemotherapy or radiotherapy are still ineffective. Thus, new therapies and drugs are required.

Research frontiers

Photodynamic therapy (PDT) has been used as adjuvant therapy in a wide range of malignant cancers, such as esophageal and skin cancer, with good curative effects. In PDT, only cells in contact with the photosensitizer (PS), light and oxygen are affected, which make it superior to other adjuvant therapies.

Innovations and breakthroughs

Quantum dots (QDs) have large absorption spectra, narrow and symmetric emission bands, and a high molar extinction coefficient, which make them superior to conventional PSs in PDT. In this study, the authors synthesized QDs with a CdSe core and a ZnS shell and demonstrated the inhibitory effect of QD-induced PDT on pancreatic cancer cells. This effect may be due to ROS generation, caspase-3 cleavage and some apoptotic molecular regulation.

Applications

In the present study, QD-induced PDT showed cytotoxicity to pancreatic cancer cells. This reveals a new potential therapeutic strategy for pancreatic cancer.

Terminology

PDT is an innovative treatment that utilizes a photosensitizing agent followed by light exposure to treat certain diseases.

Peer-review

Authors investigated photodynamic effect of CdSe/ZnS QDs on pancreatic SW1990 cancer cells, and concluded that it could be used as a photosensitizer inhibiting SW1990 cells proliferation and apoptotic protein expression regulation. The study is interesting, with convincing results and conclusions.

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ORIGINAL ARTICLE

Basic Study

Interleukin-22 ameliorates acute severe pancreatitis-associated lung injury in mice

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Author contributions: Qiao YY, Liu XQ and Xu HW designed the experiments; Qiao YY, Liu XQ, Xu CQ, Zhang Z and Xu HW performed the research; Xu CQ and Xu HW contributed new reagents/analytic tools; Qiao YY, Zhang Z and Xu HW analyzed the data; Qiao YY and Liu XQ wrote the paper; Xu CQ and Xu HW revised the manuscript.

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Institutional animal care and use committee statement: All animal experiments were carried out with approval by an ethics committee in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and further approved by the Institutional Care Committee (Shandong University, Jinan, China).

Conflict-of-interest statement: None of the authors have any possible conflicts of interest.

Data sharing statement: There are no additional data available.

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Abstract

AIM: To investigate the potential protective effect of exogenous recombinant interleukin-22 (rIL-22) on L-arginine-induced acute severe pancreatitis (SAP)-associated lung injury and the possible signaling pathway involved.

METHODS: Balb/c mice were injected intraperitoneally with L-arginine to induce SAP. Recombinant mouse IL-22 was then administered subcutaneously to mice. Serum amylase levels and myeloperoxidase (MPO) activity in the lung tissue were measured after the L-arginine administration. Histopathology of the pancreas and lung was evaluated by hematoxylin and eosin (HE) staining. Expression of B cell lymphoma/leukemia-2 (Bcl-2), Bcl-xL and IL-22RA1 mRNAs in the lung tissue was detected by real-time PCR. Expression and phosphorylation of STAT3 were analyzed by Western blot.

RESULTS: Serum amylase levels and MPO activity in the lung tissue in the SAP group were significantly higher than those in the normal control group ($P < 0.05$). In addition, the animals in the SAP group showed significant pancreatic and lung injuries. The expression of Bcl-2 and Bcl-xL mRNAs in the SAP group

was decreased markedly, while the IL-22RA1 mRNA expression was increased significantly relative to the normal control group ($P < 0.05$). Pretreatment with PBS did not significantly affect the serum amylase levels, MPO activity or expression of Bcl-2, Bcl-xL or IL-22RA1 mRNA ($P > 0.05$). Moreover, no significant differences in the degrees of pancreatic and lung injuries were observed between the PBS and SAP groups. However, the serum amylase levels and lung tissue MPO activity in the rIL-22 group were significantly lower than those in the SAP group ($P < 0.05$), and the injuries in the pancreas and lung were also improved. Compared with the PBS group, rIL-22 stimulated the expression of Bcl-2, Bcl-xL and IL-22RA1 mRNAs in the lung ($P < 0.05$). In addition, the ratio of p-STAT3 to STAT3 protein in the rIL-22 group was significantly higher than that in the PBS group ($P < 0.05$).

CONCLUSION: Exogenous recombinant IL-22 protects mice against L-arginine-induced SAP-associated lung injury by enhancing the expression of anti-apoptosis genes through the STAT3 signaling pathway.

Key words: Interleukin-22; Acute severe pancreatitis; Lung injury; Anti-apoptosis gene; Signal transducer and activator of transcription 3

Core tip: Interleukin-22 (IL-22) is recognized today as a key player in the antimicrobial defense, regeneration, and protection against damage. However, no reports have described the effects of IL-22 on acute severe pancreatitis (SAP)-associated lung injury. In this study, we found that IL-22 alleviated SAP-associated lung injury in mice by enhancing the expression of anti-apoptosis genes, such as Bcl-2 and Bcl-xL, through the STAT3 signaling pathway. Therefore, IL-22 and the components of STAT3 signaling pathway may be promising targets in the treatment of SAP-associated lung injury.

Qiao YY, Liu XQ, Xu CQ, Zhang Z, Xu HW. Interleukin-22 ameliorates acute severe pancreatitis-associated lung injury in mice. *World J Gastroenterol* 2016; 22(21): 5023-5032 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i21/5023.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i21.5023

INTRODUCTION

Acute lung injury (ALI) is the most common and serious extrapancreatic complication of acute severe pancreatitis (SAP) and also represents a dominant contribution to high morbidity and mortality rates[1]. However, the mechanism underlying the pathogenesis of SAP-induced ALI remains poorly understood. Current therapeutic approaches are limited, and predominantly aimed at symptomatic and supportive treatments. Studies have shown that SAP leads to the overproduction of several cytokines and inflammatory mediators, which initiates and amplifies systemic inflammatory response syndrome (SIRS), resulting in distant organ dysfunction and the development of ALI[2]. The unmet need for therapies against SAP-associated lung injury and paucity of immune response understanding in SAP urge us to explore the role of interleukin-22 (IL-22) and its possible signaling pathway.

IL-22 is a member of the IL-10 cytokine family with epithelial reparative and regenerative properties and is produced by T helper (Th) 22, Th1, and Th17 cells, γδ T cells, natural killer T (NKT) cells, and innate lymphoid cells (ILCs)[3]. Since its discovery in 2000[4], several research laboratories have made great progress in exploring the biology of IL-22 and the role of IL-22 has been identified in numerous tissues, such as the small intestine, liver, colon, lung, kidney, skin, thymus, and pancreas[3,5]. IL-22 exerts its functions by binding to a transmembrane receptor complex that is composed of two different subunits: IL-22 receptor subunit alpha-1 (IL-22RA1) and IL-10R2[6]. IL-22 receptor activation leads to signal transducer and activator of transcription (STAT) 3-mediated proliferative and anti-apoptotic pathway signaling, as well as antimicrobial induction that helps prevent damage and aid tissue repair[5,7]. Treatment with IL-22, *via* the activation of STAT3, alleviates tissue destruction, promotes intestinal epithelial cell proliferation and survival, and accelerates mucosal wound healing during dextran sodium sulfate (DSS)-induced colitis[8], and contributes to the recovery of goblet cell mucus and rapid amelioration of local intestinal inflammation in Th2-mediated colitis[9]. Similarly, IL-22 is a survival factor for hepatocytes in D-galactosamine (GalN)/lipopolysaccharide (LPS)-induced acute liver failure[10] and has a protective role against acute kidney injury induced by ischemia-reperfusion in mice[11]. In addition, IL-22 can also protect mice against acute pancreatitis induced by caerulein and by choline-deficient diet supplemented with DL-ethionine (CDE)[12]. However, no reports have described the effects of IL-22 on SAP-associated lung injury. The purpose of this study was to examine whether IL-22 could protect mice against SAP-associated lung injury induced by L-arginine and its possible signaling pathway.

MATERIALS AND METHODS

Experimental animals

Male Balb/c mice weighing 18-22 g were provided by the Experimental Animal Center of Shandong University (China). All animals were fed laboratory chow, given water *ad libitum*, and maintained in plastic cages at a constant temperature of 23 ℃ ± 2 ℃ and a relative humidity of 55% ± 2% under a 12 h/12 h light-dark cycle for one week prior to performing

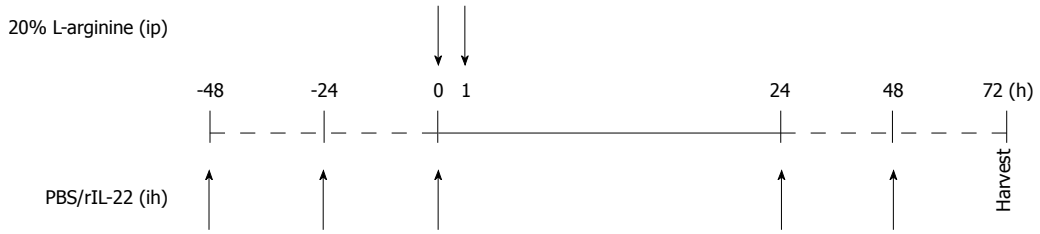

Figure 1 Time points of PBS or recombinant interleukin-22 injection. PBS: Phosphate-buffered saline; rIL-22: Recombinant interleukin-22.

the experiments. All experiments were performed according to the guidelines of the Shandong University Institutional Animal Care and Use Committee (IACUC).

Animal model of SAP and treatments

A total of 72 male Balb/c mice were deprived of food and received only water 12 h before the trial commenced. The mice were randomly assigned to four groups: normal control group (n = 12), SAP group (n = 36), treatment control group (phosphate-buffered saline (PBS) group, n = 12) and treatment group [recombinant IL-22 (rIL-22) group, n = 12]. Mice in the SAP, PBS and rIL-22 groups were injected intraperitoneally (ip) twice with 20% L-arginine hydrochloride (Sigma-Aldrich; pH = 7.0, 4 g/kg bodyweight), at an interval of 1 h. The normal control group received physiological saline injections. PBS or rIL-22 (Miltenyi Biotech) (200 ng/per, 5 times) was administered subcutaneously to mice in the PBS and rIL-22 groups (Figure 1). Mice in the SAP group were killed at 24 h, 48 h, and 72 h after the administration of L-arginine. The remaining mice were sacrificed 72 h after the L-arginine injection.

Serum amylase detection

Mice were thoroughly anesthetized with ether. Orbital blood was collected and stored at -80 ℃ until analysis. Serum amylase levels were measured using an automatic biochemical analyzer.

Determination of myeloperoxidase activity in the lung

The left upper lobe of the lung was harvested and stored at -80 ℃ until assessment. Cryopreserved tissue samples were homogenized, and myeloperoxidase (MPO) activities were measured with MPO detection assay kits following the manufacturer's instructions (Jiancheng Company, Nanjing, China). MPO, a mark of neutrophil accumulation and activation, was expressed as activity units per gram of lung tissue.

Pathological examinations

The head of the pancreas and right upper lobe of the lung were harvested from each mouse and immediately fixed in 10% buffered formalin overnight. Samples were embedded in paraffin wax and cut into 4 μm sections. The sections were flattened, mounted, and heated on blank glass slides. After deparaffinization and dehydration, the sections were stained with hematoxylin and eosin (HE). Pathological examinations were performed by a blinded, unbiased pathologist. The severity of pancreas injury was evaluated based on pancreatic tissue edema, hemorrhage, necrosis and infiltration of inflammation cells. The severity of lung injury was measured according to alveolar congestion, necrosis, hemorrhage, leucocyte infiltration, and thickness of the alveolar membrane.

Real-time polymerase chain reaction

B cell lymphoma/leukemia-2 (Bcl-2), B cell lymphoma/leukemia-extra large (Bcl-xL) and IL-22RA1 mRNA expression levels in the lung tissue were detected by real-time polymerase chain reaction (PCR). Total RNA was isolated from the frozen lung tissue using Trizol reagent (Takara Bio Inc., Japan) following the manufacturer's instructions. RNA purity was tested using a spectrophotometer (Nanodrop Technologies, Wilmington, DE). Reverse transcription-PCR amplification was performed according to the illustrations of the PrimeScript™ RT Reagent Kit (Takara Bio Inc., Japan) with genomic deoxyribonucleic acid (gDNA) Eraser (Perfect Real Time). Real-time PCR was conducted using the Lightcycler480 (Roche). The primer sequences were as follows: Bcl-2: forward, 5′-TGAAGCGGTCCGGTGGATA-3′, reverse, 5′-CAGCATTTGCAGAAGTCCTGTGA-3′; Bcl-xL: forward, 5′-GAGGCAGGCGATGAGTTG-3′, reverse, 5′-ACGATGCGACCCCAGTTT-3′; IL-22RA1: forward, 5′-TCTGGGCTACAAATACATCACCAAG-3′, reverse, 5′-GGCCACTGAGGTCCAAGACA-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward, 5′-AAATGGTGAAGGTCGGTGTGAAC-3′, reverse, 5′-CAACAATCTCCACTTTGCCACTG-3′. The cycle conditions were as follows: cDNA denaturation at 95 ℃ for 2 min, followed by 40 cycles of denaturation at 95 ℃ for 10 s, annealing at 60 ℃ for 30 s, and extension at 72 ℃ for 30 s. The specificity of the product was assessed from a melting curve analysis. Results were standardized using GAPDH, and relative amounts were then calculated according to a $2^{-\Delta\Delta CT}$ method.

Western blot analysis

Lung tissue (0.5 g) was ground rapidly in liquid nitrogen to provide 1 mL homogenate (including a protease inhibitor cocktail), which was diluted 20-fold with the radio-immunoprecipitation assay (RIPA)

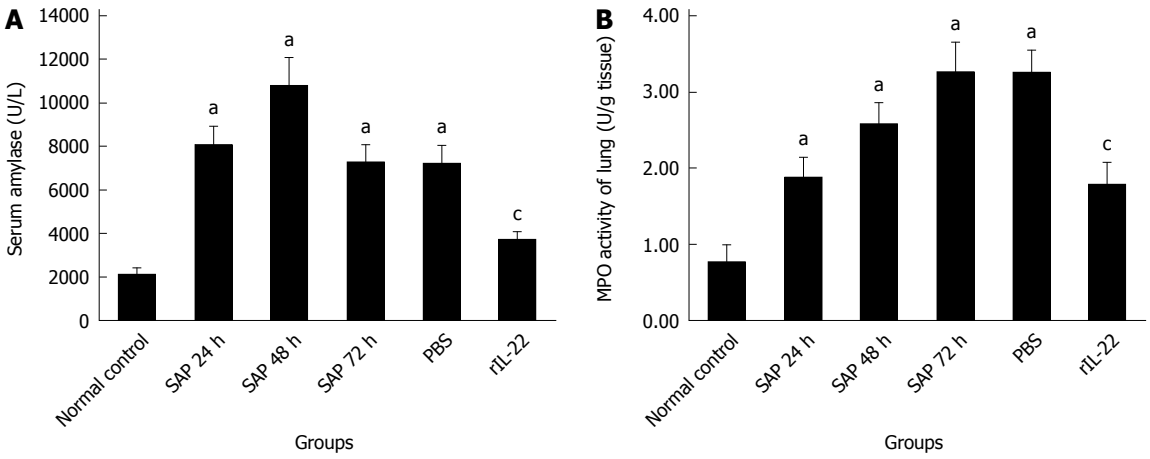

Figure 2 Serum amylase (A) and activity of lung myeloperoxidase (B). Numbers of cases from each group statistically analyzed are 12 (Normal control), 9 (SAP 24 h), 8 (SAP 48 h), 8 (SAP 72 h), 8 (PBS) and 12 (rIL-22). Results are presented as mean ± SD. $^{a}P < 0.05$ *vs* the normal control group, $^{c}P < 0.05$ *vs* the SAP group at 72 h. SAP: Acute severe pancreatitis; MPO: Myeloperoxidase; PBS: Phosphate-buffered saline; rIL-22: Recombinant interleukin-22.

efficient cracking liquid (Beyotime Biotech, China) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Biotechnology Inc., United States) after being separated on precast 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) (Beyotime Biotech, China). The membranes were blocked with 5% non-fat dry milk for 1 h. Primary rabbit monoclonal anti-STAT3 (1:2000) and phospho-STAT3^{Tyr705} (p-STAT3^{Tyr705}) (1:2000) antibodies (Cell Signaling, Beverly, MA, United States) were then added and incubated overnight on a rotating wheel at 4 ℃. The membranes were washed three times with tris-buffered saline with tween-20 (TBST) and incubated with a horseradish peroxidase-conjugated secondary antibody (1:2000) for 1 h at room temperature. Finally, the membranes were detected with an enhanced chemiluminescence reagent (ECL, Millipore Biotechnology Inc., United States). Band densities were measured using ImageJ Analysis Software. β-actin served as an internal control protein.

Statistical analysis

The data are presented as the mean ± SD and were statistically analyzed using SPSS version 20.0 software. The statistical differences between multiple groups were determined using one-way analysis of variance. Comparisons between the two groups were conducted using an unpaired *t*-test. A *P*-value < 0.05 was considered statistically significant. Statistical graphs were generated with GraphPad Prism 5 software.

RESULTS

Serum levels of amylase

As indicated in Figure 2A, compared with the normal control group, the level of serum amylase in the SAP group was increased markedly ($P < 0.05$). The highest level of serum amylase was detected at 48 h after the injection of L-arginine. At 72 h after injection, the serum amylase level partially recovered. No significant decrease was found in the PBS group relative to the SAP group at 72 h after the injection ($P > 0.05$). However, the rIL-22 group was markedly lower than SAP group at 72 h after the injection ($P < 0.05$).

Myeloperoxidase activity analysis

As demonstrated in Figure 2B, a gradual increase over time (24, 48, and 72 h after the injection of L-arginine) was observed in the lung MPO activities relative to the normal control group ($P < 0.05$). Pretreatment with PBS did not significantly affect the MPO activity compared to the SAP group at 72 h after the injection ($P > 0.05$). In contrast, rIL-22 significantly decreased the MPO activity compared with the SAP group at 72 h after the injection.

Histomorphology of the pancreas

Macroscopically, the pancreas was edematous 24 and 48 h after the L-arginine administration. By contrast, the pancreas shrank, with many saponification spots on the omentum majus and mesentery, intestinal cavity expanding and bloody ascites, 72 h after the injection of L-arginine and pretreatment with PBS. Under the light microscope, mice in the SAP group 24 h after L-arginine injection exhibited interstitial edema and infiltration of a small number of neutrophils and mononuclear cells. The acinar architecture and integrity were partially destroyed with focal parenchyma necrosis and hemorrhage. The vascular and pancreatic ductal structures appeared undamaged (Figure 3B). At 48 h, interstitial edema, inflammatory cellular infiltration, parenchyma necrosis and hemorrhage were significantly aggravated (Figure 3C). The severity of pancreatic destruction became maximal 72 h after L-arginine administration. About 70%-80% of the pancreatic acinar cells had been destroyed and replaced by inflammatory and fibrotic cells. The pancreatic ducts were expanded and appeared more

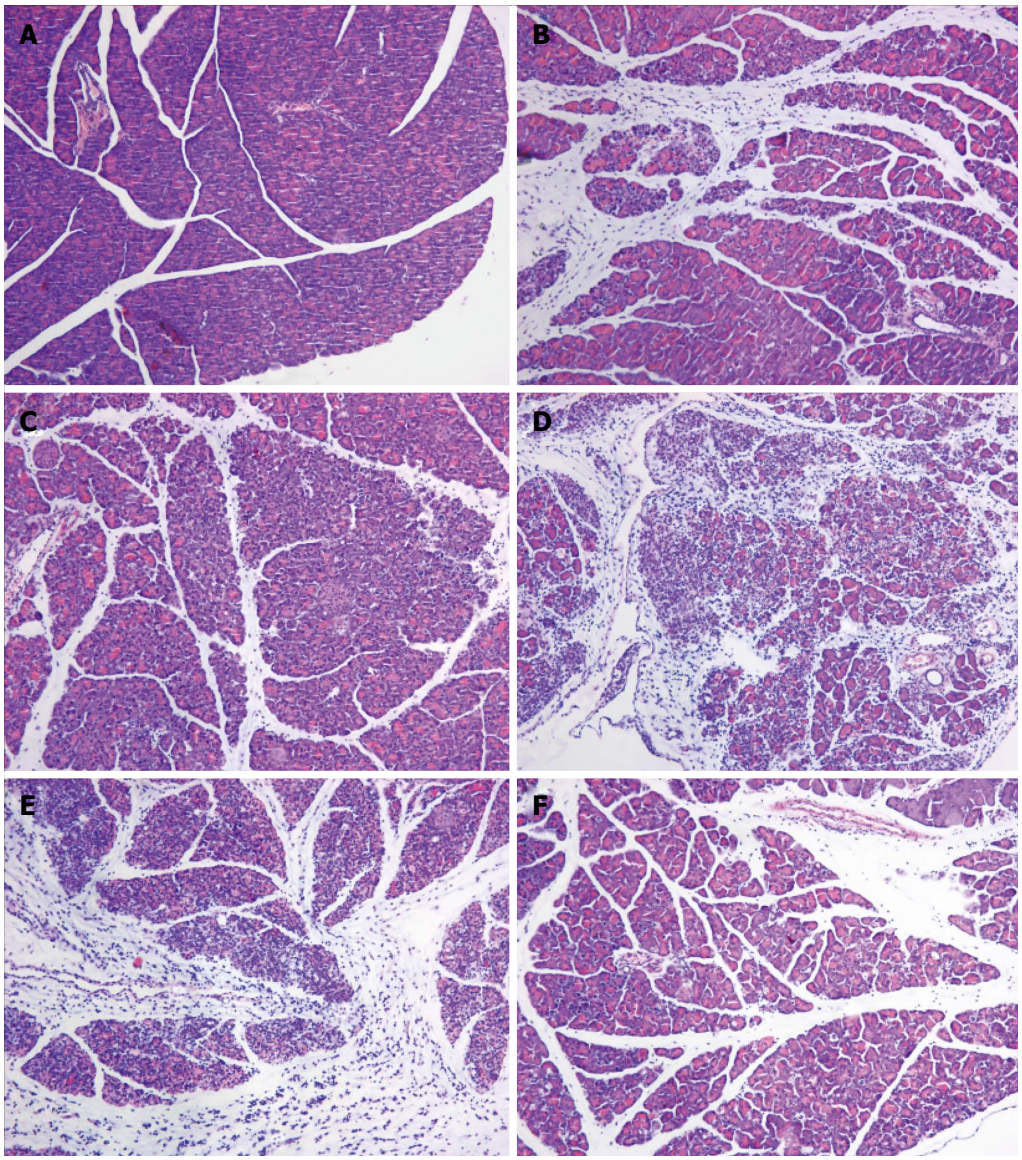

Figure 3 Hematoxylin and eosin staining of the pancreas. Normal control group (A), SAP group (B: 24 h after L-arginine injection; C: 48 after L-arginine injection; D: 72 h after L-arginine injection), PBS group (E) and rIL-22 group (F). Original magnification × 200. SAP: Acute severe pancreatitis; PBS: Phosphate-buffered saline; rIL-22: Recombinant interleukin-22.

numerous because of a decrease in acini and shrinkage of the pancreatic tissue (Figure 3D). Compared with the SAP group, PBS did not significantly reduce the pancreatic injury (Figure 3E). However, the mice in the rIL-22 group showed milder interstitial edema, acinar cell necrosis and cellular infiltration (Figure 3F).

Histomorphology of the lung

The macroscopic view of the lung showed significant edema and hemorrhage in the SAP and PBS groups. By contrast, pulmonary edema and hemorrhage in the rIL-22 group were not obvious. H&E staining showed no observable signs of lung damage in the normal control group (Figure 4A). The lungs of the mice showed no significant swelling, inflammation or necrosis 24 h and 48 h after the L-arginine injection (Figure 4B and D). However, interstitial edema, patchy hemorrhage, thickened alveolar interstitium and infiltration of inflammatory cells were markedly observed 72 h after the L-arginine injection (Figure 4D). No significant difference in the degree of lung injury was found between the PBS and SAP groups at 72 h (Figure 4E). In contrast, pretreatment with rIL-22 significantly reduced the degrees of edema, alveolar congestion and infiltration of inflammatory cells compared with the SAP group at 72 h (Figure 4F).

Bcl-2, Bcl-xL and IL-22R1 mRNA expression in lung tissue

Compared with the normal control group, the expression of Bcl-2 and Bcl-xL mRNAs in the lung tissue in the SAP group showed a significant decrease at 48 h and 72 h after the L-arginine injection ($P < 0.05$). However, no significant decrease was found at 24

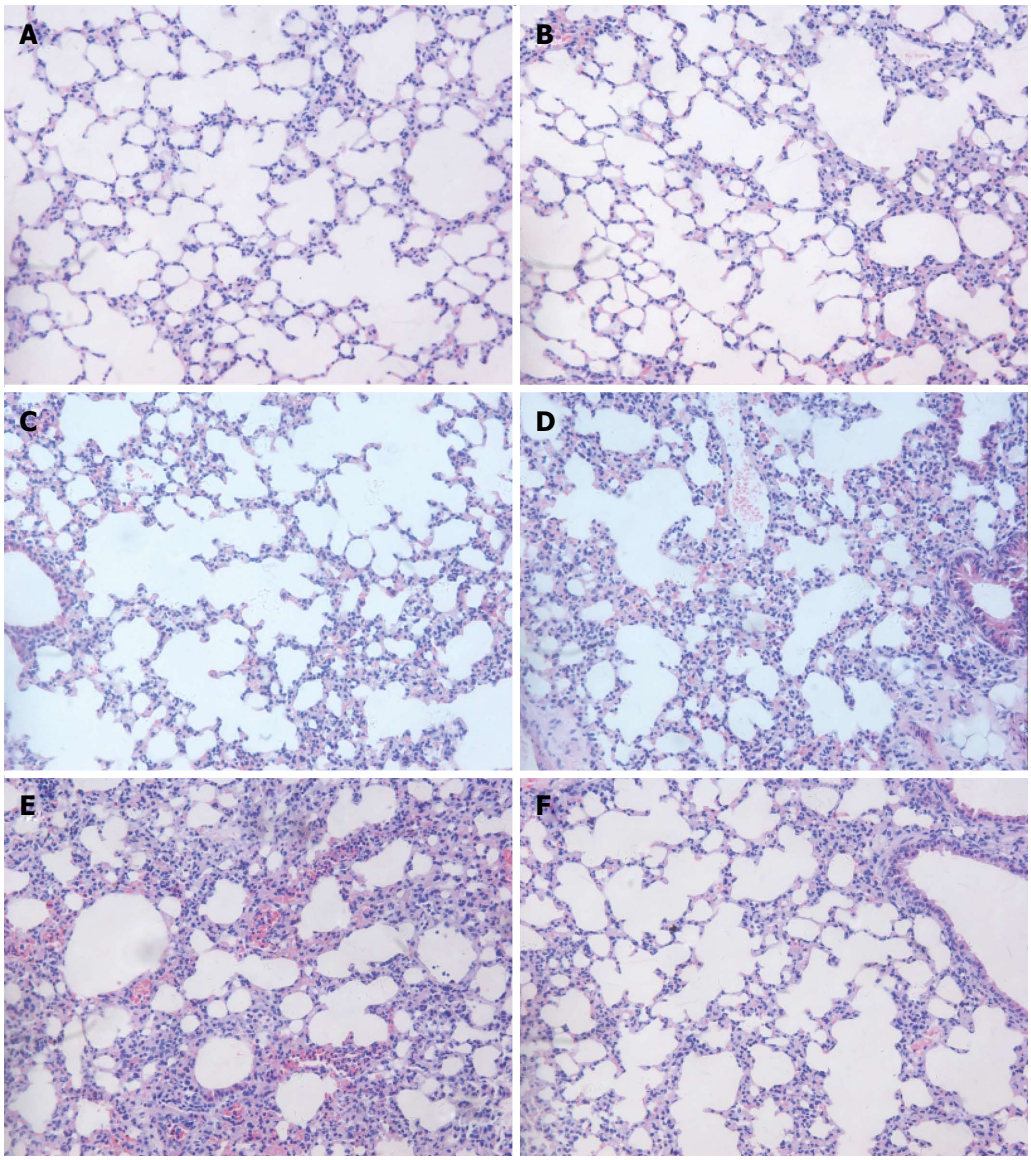

Figure 4 Hematoxylin-eosin staining of the lung. Normal control group (A), SAP group (B: 24 h after L-arginine injection; C: 48 h after L-arginine injection; D: 72 h after L-arginine injection), PBS group (E) and rIL-22 group (F). Original magnification × 200. SAP: Acute severe pancreatitis; PBS: Phosphate-buffered saline; rIL-22: Recombinant interleukin-22.

h after the injection relative to the normal control group ($P > 0.05$, Figure 5A and B). IL-22RA1 mRNA expression significantly increased in the SAP group relative to that in the normal control group ($P < 0.05$). The lowest expression level was detected at 48 h after the L-arginine injection (Figure 5C).

Exogenous IL-22 promotes lung anti-apoptosis gene expression

As demonstrated in Figure 5D, rIL-22 stimulated the expression of Bcl-2 and Bcl-xL mRNAs, which play a key role in preventing cells from apoptosis involved in tissue regeneration ($P < 0.05$). In addition, IL-22RA1 expression in the rIL-22 group was also significantly higher than that in the PBS group ($P < 0.05$).

STAT3 is involved in the protective role of IL-22 in SAP-associated lung injury

STAT3 activation in the lung tissue in rIL-22 group was higher than that in the PBS group. However, there was no significant difference between the two groups as to the STAT3 expression (Figure 6A). The ratio of p-STAT3 to STAT3 protein in the rIL-22 group was significantly higher than that of the PBS group ($P < 0.05$, Figure 6B).

DISCUSSION

SAP is a life-threatening disease characterized by obvious inflammatory reactions and its morbidity has been reported to be increasing continually in

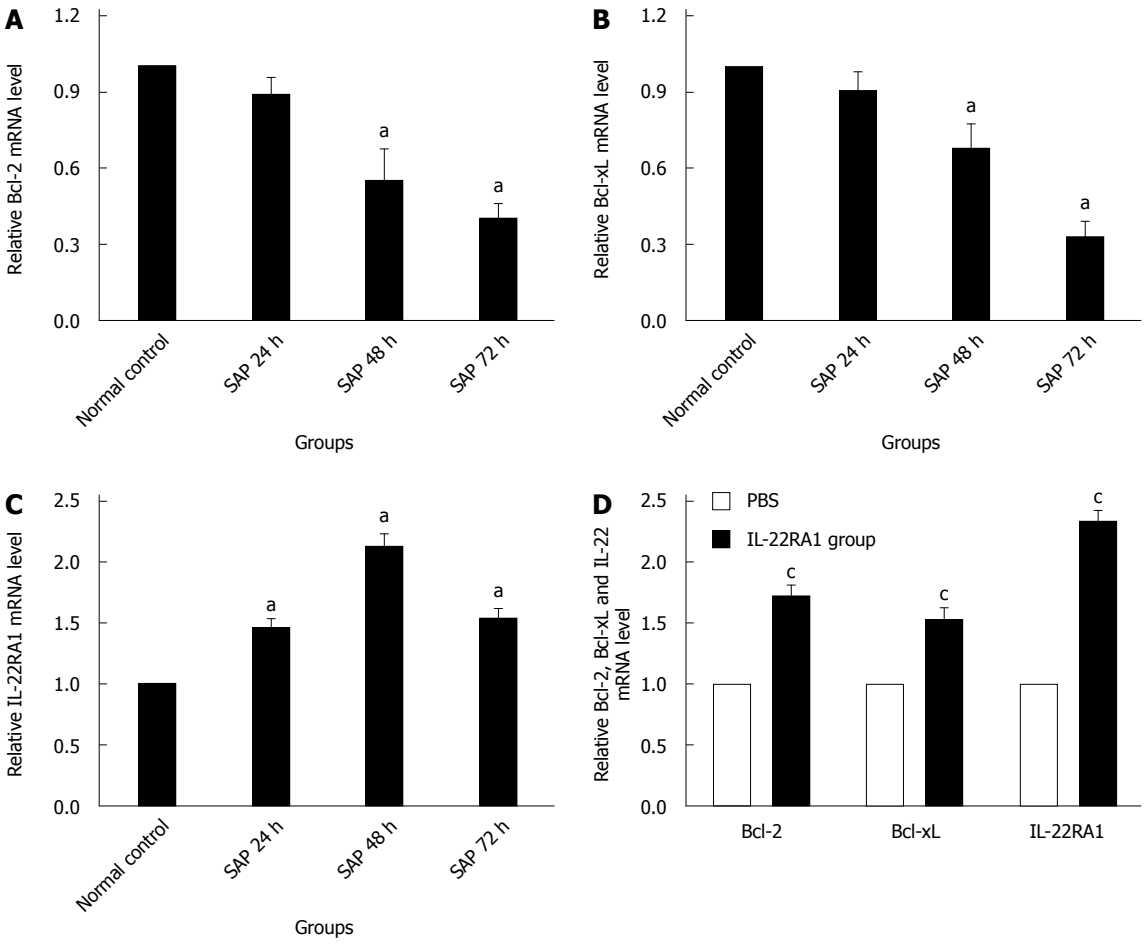

Figure 5 Real-time PCR analysis of expression of Bcl-2(A and D), Bcl-xL (B and D) and IL-22RA1 mRNAs (C and D) in lung tissue. Numbers of cases from each group statistically analyzed are 12 (Normal control), 9 (SAP 24 h), 8 (SAP 48 h), 8 (SAP 72 h), 8 (PBS) and 12 (rIL-22). Results are presented as mean ± SD. $^{a}P < 0.05$ *vs* the normal control group, $^{c}P < 0.05$ *vs* the PBS group. Bcl-2: B cell lymphoma/leukemia-2; Bcl-xL: B cell lymphoma/leukemia-extra large; IL-22RA1: Interleukin-22 receptor subunit alpha-1; PBS: Phosphate-buffered saline; rIL-22: Recombinant interleukin-22; STAT3: Signal transducer and activator of transcription 3.

recent years. Acinar necrosis-induced inflammation can lead to the occurrence of SIRS at an early stage which determines the severity of acute pancreatitis and can induce multiple organ dysfunction syndrome (MODS). ALI is the most prominent extra-pancreatic complication of SAP, and its severity ranges from mild hypoxemia to severe acute respiratory distress syndrome (ARDS) which is responsible for the high mortality rate[1,13]. Recent studies have indicated that the activation of numerous inflammatory cells, such as neutrophils and macrophages, leads to excessive production of cytokines and inflammatory mediators, regulating the severity of acute pancreatitis and SAP-associated ALI[2,14]. An ideal model for studying SAP and its associated multiple organ dysfunction should resemble the course in the human clinical setting, be easily reproducible and severe enough to induce MODS, yet still has a time window long enough for an intervention. In our experiment, the mouse SAP model induced by the L-arginine injection is compatible with the clinical manifestations of SAP, including lung damage. The serum amylase levels and lung tissue MPO activity were increased significantly after the L-arginine administration. In addition, the animals in the SAP group showed obvious pancreatic and lung injuries.

IL-22, formerly named IL-10-related T–cell-derived inducible factor (IL-TIF)[4], has recently gained considerable interest for its tissue protective effects in several murine models. At present, most studies support a well-established protective role for IL-22 in the prevention of hepatocellular damage. Specifically, application of recombinant IL-22 remarkedly attenuates murine liver injury induced by concanavalin A, alcohol, acetaminophen, hepatectomy or ischemia-reperfusion[15-19]. Furthermore, provision of IL-22 also ameliorates high fat diet-induced liver lipogenesis and hepatic steatosis[20] and decreases hepatic fibrosis in mice[21]. In addition to the protective effects of IL-22 on the liver, the benefits of IL-22 application on other targets are also well testified. Administration of IL-22 contributes to the tissue protection and regeneration in murine models of mucocutaneous infection[22-25], ventilator-induced lung injury[26], renal ischemia-reperfusion injury[27] and inflammatory bowel disease[28]. However, it was unknown whether IL-22

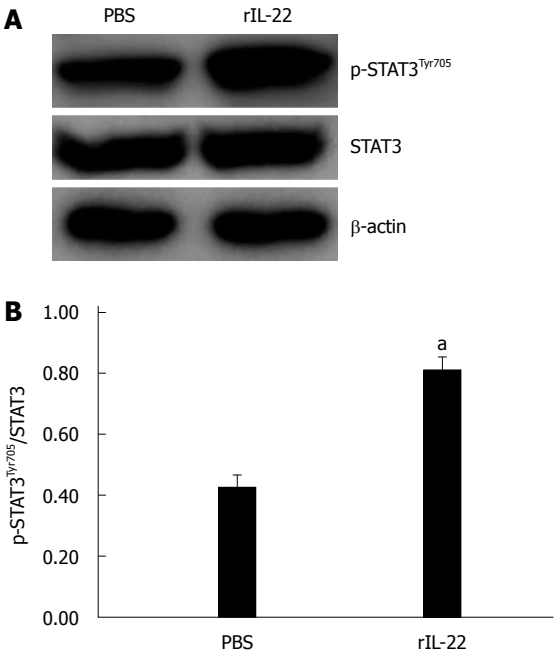

Figure 6 Western blot analysis of expression and activation (Tyr705 phosphorylation) of STAT3 protein in lung tissue (A) and the ratio of p-STAT3^{Tyr705} to STAT3 protein (B). Numbers of cases from each group statistically analyzed were 8 (PBS) and 12 (rIL-22). Results are presented as mean ± SD. $^{a}P < 0.05$ *vs* the PBS group. PBS: Phosphate-buffered saline; rIL-22: Recombinant interleukin-22; STAT3: Signal transducer and activator of transcription 3.

also plays a protective role in SAP-induced ALI. In the present study, we found that the rIL-22-treated mice had significantly lower MPO activity in the lungs than in the untreated SAP mice. Moreover, when compared to the untreated SAP mice, the rIL-22-treated mice showed significantly reduced degrees of edema, alveolar congestion, thickness of the alveolar wall and infiltration of inflammatory cells in the lung injury. Our results are in agreement with the previous findings and demonstrate that rIL-22 may be beneficial to the recovery of SAP-associated lung injury induced by L-arginine.

IL-22 is an important cytokine allowing for cross-talk between leukocytes and epithelial cells because IL-22 production and receptor expression are restricted to leukocytes and epithelial cells, respectively[3,29]. Ligation of the IL-22-IL-22R1-IL-10R2 complex leads to the activation of the JAK1/Tyk2/STAT3 pathway which is known as the principal and dominant mechanism of cellular activation by IL-22[7,8]. Of the cellular tasks associated with STAT3, anti-apoptosis, proliferation and regeneration are the major biological properties of IL-22[30,31]. Apoptosis is recognized as "programmed cell death". Excessive apoptosis probably leads to lung dysfunction in pathological situations. There are increasing data indicating that increased epithelial/endothelial cell apoptosis is involved in the pathogenesis of ALI[32-34]. The signal transduction mechanisms of apoptosis are very complex, and the anti-apoptosis pathway is associated with genes whose functions include "death receptors" and apoptotic regulation. Apoptosis-inducing genes include p53, factor associated suicide (Fas), interlukin-1β-converting enzyme (ICE), reaper (rpr), Bcl-xs, Bcl-2 associated X protein (Bax), Bcl-2 homologous antagonist killer (Bak), Bcl-2/ Bcl-2-XL-associated death promoter (Bad), Bcl-2 inhibitory BH3 domain-containing protein (bid), and Bcl-2 interacting killer (bik). Genes that inhibit apoptosis include Bcl-2, the inhibitor of apoptosis protein (IAP) family, Bcl-xl, Bcl-2 related protein A1 (A1)/Bfl1, Bcl-w, myeloid cell leukemia (Mcl), and Bcl-2-associated athanogene 1 (BAG-1)[35,36]. Bcl-2 is involved in the transduction of the intrinsic mitochondria pathway[37]. IL-22 up-regulates STAT3-inducible proteins, such as Bcl-2, Bcl-xL, cyclin-dependent kinase 4 (CDK4), cyclin D1, cellular-myelocytomatosis viral oncogene (*c-myc*), and p21[15,19,21,38-40], and is associated with tissue protection and regeneration from injury. Along with the activation of extracellular regulated protein kinases (ERK) 1/2[41] and Akt/protein kinase B (PKB)[42], these pro-survival proteins are likely to form the cellular basis for tissue protective characteristics of IL-22. In this study, we examined the expression of IL-22RA1 in the lung of mice with SAP. Interestingly, the level of IL-22R1 expression increased at first and decreased later after the L-arginine administration. This finding indicates that IL-22RA1 expression is stress inducible. Based on the high expression of IL-22RA1, we hypothesized that the lung would respond strongly to an IL-22 administration. To test this hypothesis, we administered rIL-22 systemically to mice and assessed the IL-22 downstream signaling pathway. Consistent with our hypothesis, the lung expression of Bcl-2 and Bcl-xL, downstream targets of STAT3 activation, was decreased significantly in mice with SAP, but was interestingly elevated after rIL-22 treatment. Similarly, IL-22RA1 was also increased after rIL-22 administration. In addition, significant STAT3 activation was observed in the lung after the exogenous IL-22 administration. These data indicate that the mice with SAP-associated lung injury were responsive to rIL-22 administration by enhancing the expression of anti-apoptosis genes through the STAT3 signaling pathway.

Taken together, these findings indicate that systemic administration of rIL-22 can alleviate the SAP-associated ALI in mice by enhancing the expression of anti-apoptosis genes, such as Bcl-2 and Bcl-xL. While the underlying mechanism of IL-22 in protection against SAP-associated lung injury is not fully understood, our findings demonstrate that the STAT3 signaling pathway may be associated with this process. Therefore, IL-22 and the components of STAT3 signaling pathway may be promising targets in the treatment of SAP-associated lung injury.

COMMENTS

Background

Interleukin-22 (IL-22) is recognized today as a key player in the antimicrobial

defense, regeneration, and protection against damage. However, no reports have described the effects of IL-22 on acute severe pancreatitis associated lung injury. In this article, the authors sought to investigate the potential protective effect of exogenous rIL-22 on SAP associated lung injury induced by L-arginine and its possible signaling pathway.

Research frontiers

IL-22 is recognized today as a key player in the antimicrobial defense, regeneration, and protection against damage. However, no reports have described the effects of IL-22 on acute severe pancreatitis associated lung injury.

Innovations and breakthroughs

In this article, the authors investigated the potential protective effect of exogenous rIL-22 on SAP associated lung injury induced by L-arginine and its possible signaling pathway. IL-22 was demonstrated to alleviate acute severe pancreatitis-associated acute lung injury in mice by enhancing the expression of anti-apoptosis genes such as Bcl-2 and Bcl-xL through the STAT3 signaling pathway.

Applications

IL-22 and the components of STAT3 signaling pathway may be promising targets in the treatment of acute severe pancreatitis associated lung injury.

Terminology

IL-22, a member of the IL-10 family, is a cytokine secreted by several types of immune cells such as T helper (Th) 22, Th1, and Th17 cells, γδ T cells, natural killer T cells, and innate lymphoid cells. It is a principal component in mucosal barrier defense, tissue repair, epithelial cell survival, and proliferation.

Peer-review

The authors show that recombinant IL-22 protected the mice against L-arginine-induced SAP and associated lung injury by enhancing the expression of anti-apoptosis genes. The work is very innovative and is an indication for further research on the problem of complications of acute pancreatitis.

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ORIGINAL ARTICLE

Basic Study

^{125}I-labeled anti-bFGF monoclonal antibody inhibits growth of hepatocellular carcinoma

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Abstract

AIM: To investigate the inhibitory efficacy of ^{125}I-labeled anti-basic fibroblast growth factor (bFGF) monoclonal antibody (mAb) in hepatocellular carcinoma (HCC).

METHODS: bFGF mAb was prepared by using the 1G9B9 hybridoma cell line with hybridization technology and extracted from ascites fluid through a Protein G Sepharose affinity column. After labeling with ^{125}I through the chloramine-T method, bFGF mAb was further purified by a Sephadex G-25 column. Gamma radiation counter GC-1200 detected radioactivity of ^{125}I-bFGF mAb. The murine H22 HCC xenograft model was established and randomized to interventions with control (phosphate-buffered saline), ^{125}I-bFGF mAb,

^{125}I plus bFGF mAb, bFGF mAb, or ^{125}I. The ratios of tumor inhibition were then calculated. Expression of bFGF, fibroblast growth factor receptor (FGFR), platelet-derived growth factor, and vascular endothelial growth factor (VEGF) mRNA was determined by quantitative reverse transcriptase real-time polymerase chain reaction.

RESULTS: The purified bFGF mAb solution was 8.145 mg/mL with a titer of 1:2560000 and was stored at -20 ℃. After coupling, ^{125}I-bFGF mAb was used at a 1: 1280000 dilution, stored at 4 ℃, and its specific radioactivity was 37 MBq/mg. The corresponding tumor weight in the control, ^{125}I, bFGF mAb, ^{125}I plus bFGF mAb, and ^{125}I-bFGF mAb groups was 1.88 ± 0.25, 1.625 ± 0.21, 1.5 ± 0.18, 1.41 ± 0.16, and 0.98 ± 0.11 g, respectively. The tumor inhibition ratio in the ^{125}I, bFGF mAb, ^{125}I plus bFGF mAb, and ^{125}I-bFGF mAb groups was 13.6%, 20.2%, 25.1%, and 47.9%, respectively. Growth of HCC xenografts was inhibited significantly more in the ^{125}I-bFGF mAb group than in the other groups (P < 0.05). Expression of bFGF and FGFR mRNA in the ^{125}I-bFGF mAb group was significantly decreased in comparison with other groups (P < 0.05). Groups under interventions revealed increased expression of VEGF mRNA (except for ^{125}I group) compared with the control group.

CONCLUSION: ^{125}I-bFGF mAb inhibits growth of HCC xenografts. The coupling effect of ^{125}I-bFGF mAb is more effective than the concomitant use of ^{125}I and bFGF mAb.

Key words: Basic fibroblast growth factor; ^{125}Iodine; Monoclonal antibody; Hepatocellular carcinoma; Fibroblast growth factor receptor; Vascular endothelial growth factor

Core tip: The aim of this study was to investigate the inhibitory efficacy of ^{125}I-basic fibroblast growth factor (bFGF) monoclonal antibody (mAb) in mice with hepatocellular carcinoma (HCC). ^{125}I-bFGF mAb inhibited the growth of HCC xenografts (P < 0.05). The combination of ^{125}I and bFGF mAb was more effective than the concomitant use of ^{125}I and bFGF mAb. ^{125}I-bFGF mAb also significantly reduced the expression of bFGF and fibroblast growth factor receptor (FGFR) mRNA (P < 0.05). Moreover, ^{125}I-bFGF mAb downregulated platelet-derived growth factor mRNA and upregulated vascular endothelial growth factor mRNA.

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INTRODUCTION

Hepatocellular carcinoma (HCC) ranks among the most common cancers worldwide. It is the third leading cause of cancer death, with about 700000 cases diagnosed annually[1]. It is characterized by rapid progression, metastasis, and recurrence. Surgical resection and liver transplantation are traditional therapeutic approaches for HCC. Liver transplantation offers many benefits for HCC, but shortage of donor organs and high costs constrain its application. New therapeutic methods, such as radiofrequency ablation, transcatheter arterial chemoembolization, local hyperthermia, and targeted therapy, can also be beneficial to patients with HCC[2-4].

HCC is one of the most vascularized solid tumors, and angiogenesis plays a pivotal role in its development, progression, and metastasis. Basic fibroblast growth factor (bFGF) is one of the most prominent angiogenesis-promoting agents, and its expression closely correlates with tumor angiogenesis[5]. Previous studies have revealed that bFGF stimulates proliferation of human HCC cell lines[6], and the serum bFGF levels in patients with HCC are significantly higher than those in healthy volunteers[7]. These increases in serum bFGF levels correlate closely with HCC invasion and recurrence[8,9]. These studies indicate that specific targeting of bFGF may provide a novel therapeutic strategy for HCC.

bFGF monoclonal antibody (mAb) can specifically bind to bFGF and block its growth-stimulating activity. In our previous studies, we found that bFGF mAb combined with S-1 (gimeracil and oteracil potassium) synergistically inhibited Lewis-transplanted lung cancer, which was related to its inhibition of proliferation and angiogenesis[10]. Combination of bFGF mAb and radiotherapy was shown to exert a synergistic inhibitory effect on the growth of B16-transplanted melanoma tumors, since it increases the radiosensitivity of tumor cells by reducing the expression of bFGF, decreasing angiogenesis, and promoting apoptosis[11]. bFGF mAb also inhibits the proliferation of MCF-7/ADM breast cancer cells and reverses multidrug resistance. The phenomenon may be associated with downregulation of P-glycoprotein and increased intracellular concentration of chemotherapeutic drugs[12].

^{125}I radiotherapy enhances DNA damage, and consequently, induces liver cancer cell apoptosis and improves overall survival in HCC[13]. The use of radionuclide labels on mAbs enhances the specificity of their targeting, and increases the accuracy of evaluating therapeutic response[14]. Thus, coupling bFGF mAb with ^{125}I was used in the present study. Our previous study demonstrated that the half-life of ^{125}I-bFGF mAb

was 81.6-90.3 h and that the radioactive counts were highly detected in the liver tissue of mice[15]. Therefore, ^{125}I-bFGF mAb may be an attractive therapeutic modality for HCC. In this study, we aimed to investigate the feasibility and therapeutic efficacy of ^{125}I-bFGF mAb in HCC.

MATERIALS AND METHODS

Production of bFGF mAb

We prepared the 1G9B9 hybridoma cell line, which was developed in our laboratory with hybridization technology and can secrete mAbs against bFGF. After injecting 10^5 hybridoma cells into each BABL/c mice with incomplete Freund's adjuvant (Sigma-Aldrich, St Louis, MO, United States), ascites was formed in mice 7 d later. The ascites fluid was extracted and purified twice in ammonium sulfate and a Protein G Sepharose affinity column (General Electric, Fairfield, CT, United States). bFGF mAb was identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The concentration and titer of purified bFGF mAb stock solution were assayed by bicinchoninic acid (BCA) standard assay kit (Pierce, Rockford, IL, United States) and indirectly by enzyme linked immunosorbent assay (ELISA), respectively. Finally, bFGF mAb stock solution was cryopreserved at -20 ℃.

Production of ^{125}I-bFGF mAb

bFGF mAb was labeled with ^{125}I (Amersham Biosciences, Chalfont St. Giles, United Kingdom) by using the chloramine-T method. Afterwards, ^{125}I-bFGF mAb was purified by Sephadex G-25 column (Pharmacia, Piscataway, NJ, United States) in phosphate buffered saline (PBS) (0.05 mol/L, pH 7.5) at room temperature. The labeling efficiency and titer of ^{125}I-bFGF mAb were tested by paper chromatography and indirectly by ELISA, respectively. In order to investigate the stability and storage temperature of ^{125}I-bFGF mAb, assays for radiochemical purity of ^{125}I-bFGF mAb were performed using a gamma radiation counter GC-1200 (Zhongjia Photoelectric Instrument Company, Hefei, China) in 1-8 d with variable temperatures. The radioactive counts of quality controlled samples (0.5, 5.0 and 50.0 ng/mL) were tested by gamma radiation counter GC-1200 in six replicates on three different days to evaluate the accuracy of the assay. The intra-day coefficient of variation (CV) and inter-day CV were also calculated.

Establishment of murine H22 HCC xenograft model

We adjusted the concentration of H22 hepatoma cells to 2.5 × 10^6/mL during the logarithmic growth phase. Each C57BL/6 mouse was injected with 0.2 mL of cells in the armpit of the right front limb. After the tumor diameters grew to 7-8 mm, *Kalium jodatum* was consumed by mice for 3 d to inhibit the absorption of ^{125}I by the thyroid gland before treatment. Twenty-five mice were randomized into five groups: control (PBS), ^{125}I, bFGF mAb, ^{125}I plus bFGF mAb, and ^{125}I-bFGF mAb. The injection doses for each group per mouse were 0.2 mL PBS, 7.4 MBq Na^{125}I, 200 μg bFGF mAb, 7.4 MBq Na^{125}I plus 200 μg bFGF mAb, and 37 MBq/mg ^{125}I-bFGF mAb 200 μg, respectively. The drug was given once every 3 d, five times in total (15 d). After sacrificing the mice and dissecting the tumors, the volume and weight of the tumor were measured and the ratio of tumor inhibition was calculated.

Quantification of bFGF, FGFR, VEGF, and PDGF mRNA expression by quantitative reverse transcriptase real-time polymerase chain reaction

Expression of bFGF, vascular endothelial growth factor (VEGF), fibroblast growth factor receptor (FGFR), and platelet-derived growth factor (PDGF) mRNA was measured by quantitative reverse transcriptase real-time polymerase chain reaction (qRT-PCR). β-actin was used as an internal reference gene. Total RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, United States). The concentration and quality of the extracted RNA were detected on the measured absorbance at 260 nm and a ratio of (A260/A280). cDNA was synthesized using Transcript High Fidelity cDNA Synthesis Kit (Fermentas, Waltham, MA, United States). The primers of DNA sequences were as follows: bFGF (5'-TAT TTC TTT GGC TGC TAC TTG-3' and 5'-TCC AGC ATT TCG GTG TTG-3'); FGFR (5'-CCT CGT TTG GAG ACG BCT TCA-3' and 5'-GAG CAA AGG GTG TGT GGA CTC T-3'); VEGF (5'-GAA TGT GAT TGC TTT CCT GGG TA-3' and 5'-AGT AAA AGT GGC TGT GGT GGT CCT GA-3'); PDGF (5'-GAG ATA GAC TCC GTA GGG GCT GA-3' and 5'-GAG CAA AGG GTG TGT GGA CTC T-3'); β-actin (5'-CAA GAT CAT TGC TCC TCC TGA-3' and 5'-AGT CCG CCT AGA AGC ATT TG-3'). Using Light Cycler 480 SYBR Green I Master Mix (Roche, Basel, Switzerland), qPCR was performed according to the qPCR protocol. Conditions used for the qPCR amplification were shown as follows: 95 ℃ for 5 min, 55 cycles; 94 ℃ for 10 s, 62 ℃ for 15 s, 72 ℃ for 10 s, and 65 ℃ for 1 min. Melting curves were analyzed to detect the specificity of qPCR products. The expressions of bFGF, VEGF, FGFR, and PDGF mRNA were analyzed by Mx Pro QPCR software version 3.0, and the housekeeping gene β-actin was used as a normalized target gene.

Animal care and use statement

All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Laboratory Animal Center of Jinan University. The animal protocol in our experiment was designed to minimize pain and discomfort to the mice. The mice were acclimatized to laboratory conditions (23 ℃, 12 h/12 h light/dark, 50% humidity, *ad libitum* access to food and water) for 2 wk prior to experimentation. Intragastric administration was carried out with conscious mice, using straight gavage

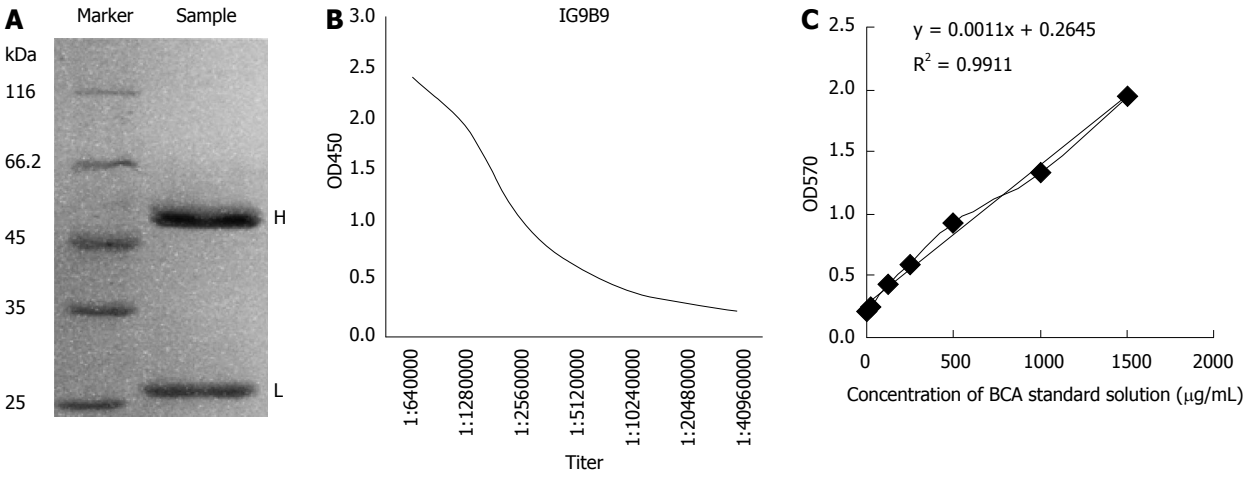


Figure 1 Characterization of anti-bFGF mAb. A: SDS-PAGE of purified bFGF mAb (H: heavy chain; L: light chain); B: Titer of purified bFGF mAb stock solution tested by indirect ELISA; C: Concentration of purified bFGF mAb stock solution assayed by BCA standard assay kit. (Standard curve: y = 0.0011x + 0.2645; Relevancy: R^2 = 0.9911). BCA: Bicinchoninic acid; bFGF: Basic fibroblast growth factor; ELISA: Enzyme-linked immunosorbent assay; mAB: Monoclonal antibody; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

needles appropriate for the animal size (15-17 g body weight: 22 gauge, 2.54 cm length, and 1.25 mm ball diameter). All mice were euthanized by barbiturate overdose (intravenous injection, 150 mg/kg pentobarbital sodium) for HCC xenograft collection.

Statistical analysis

The descriptive data are given as mean and standard deviation. The results were analyzed by SPSS version 16.0 (Chicago, IL, United States) with a *t* test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Product of bFGF mAb

Ascites was produced after 1G9B9 hybrid tumor cells were injected into the abdominal cavity of mice for 7-12 d. Each mouse provided 1.8-2.2 mL ascites fluid, and a final volume of 30 mL was obtained. Based on SDS-PAGE of purified bFGF mAb, there were only two bFGF mAb chains, and there was no non-specific chain, indicating the high purity of bFGF mAb. The molecular weight of the heavy chain was about 50 ku while the light chain was about 25 ku (Figure 1). The titer and concentration of purified bFGF mAb solution were 1:2560000 and 8.145 mg/mL, respectively (Figure 1).

Product of ^{125}I-bFGF mAb

The optimal labeling conditions for the chloramine-T method in our study consisted of chloramine-T 50 μg, Na^{125}I 3.7 MBq, $Na_2S_2O_5$ 100 μg, and bFGF mAb 100 μg, with a reaction time of 45 s. Gamma radiation counter GC-1200 was used to test the radioactivity of the collected tubes. From tubes 1-19, the radioactive counts were close to zero. Starting from tube 20, the radioactivity counts increased and peaked at tube 30. Subsequently, the counts began to decline and reached zero again at tube 46 (Figure 2). Formation of the radioactive peak indicated successful preparation of ^{125}I-bFGF mAb. The remaining liquid was abandoned. The labeling efficiency of ^{125}I-bFGF mAb was ⩾ 90%, which was tested by paper chromatography. The purity of ^{125}I-bFGF mAb became ⩾ 98% after purified by Sephadex G-25 column. The titer was 1:1280000, which implicated no decrease in immunoreactivity (Figure 2). ^{125}I-bFGF mAb was prone to denaturation at room temperature and iodine removal at -20 ℃. ^{125}I-bFGF mAb was stably maintained when stored at 4 ℃ as the level of radiochemical purity remained ⩾ 90% over 6 d. The intra-day CV of quality controlled samples (0.5, 5.0, and 50.0 ng/mL) at the radioactive counts were 0.8%, 1.3%, and 6.8%, respectively, and the inter-day CV was 4.8%, 3.7%, and 8.5%, respectively. The specific radioactivity of the ^{125}I-bFGF mAb used in this study was 37 MBq/mg.

Inhibitory efficacy of ^{125}I-bFGF mAb on HCC

The corresponding volume and weight of the tumor in the control, ^{125}I, bFGF mAb, ^{125}I plus bFGF mAb, and ^{125}I-bFGF mAb groups were 9968 ± 430, 8987 ± 360, 8217 ± 301, 7927 ± 329, and 6210 ± 298 mm^3 and 1.88 ± 0.25, 1.63 ± 0.21, 1.50 ± 0.18, 1.41 ± 0.16, and 0.98 ± 0.11 g, respectively. When compared with the control group, the tumor inhibition ratio in the ^{125}I, bFGF mAb, ^{125}I plus bFGF mAb, and ^{125}I-bFGF mAb groups was 13.6%, 20.2%, 25.1%, and 47.9%, respectively (Figure 3). ^{125}I-bFGF mAb effectively inhibited the growth of HCC ($P < 0.05$), and the tumor inhibition ratio of the ^{125}I-bFGF mAb group was higher than that in the other groups.

Quantitative changes in bFGF, FGFR, VEGF, and PDGF mRNA expression

qRT-PCR amplification and melt curves of β-actin, bFGF, FGFR, VEGF, and PDGF are shown in Figure 4. Expression of these genes entered the plateau of

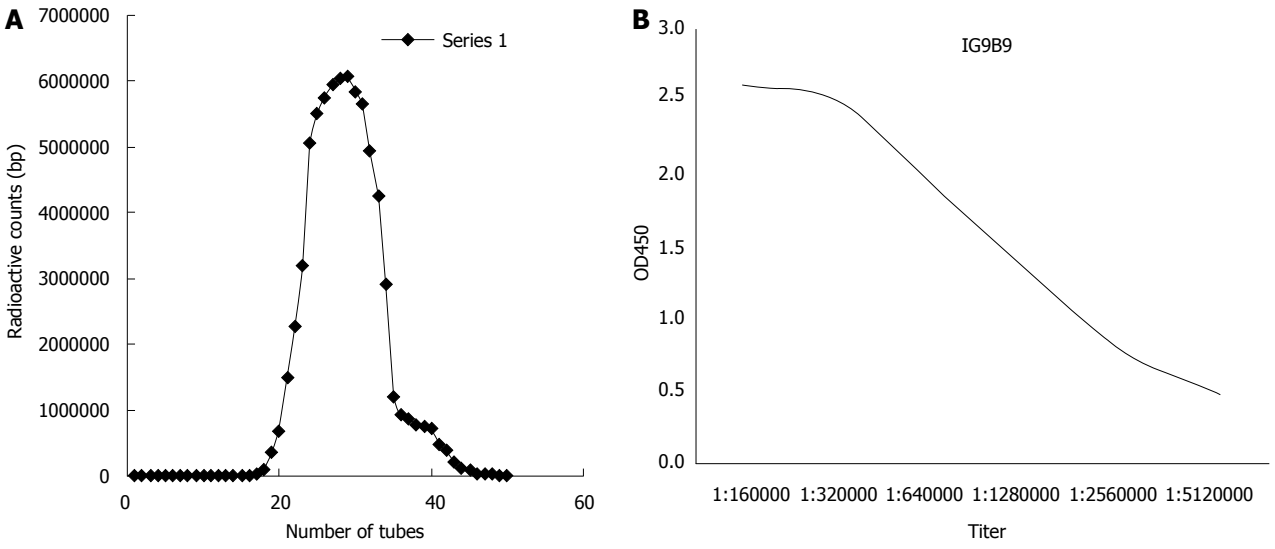


Figure 2 Radioactivity and titer of purified ^{125}I-bFGF mAb. A: Product peak of purified ^{125}I-bFGF mAb tested by gamma radiation counter GC-1200; B: Titer of purified ^{125}I-bFGF mAb stock solution assayed by indirect ELISA.

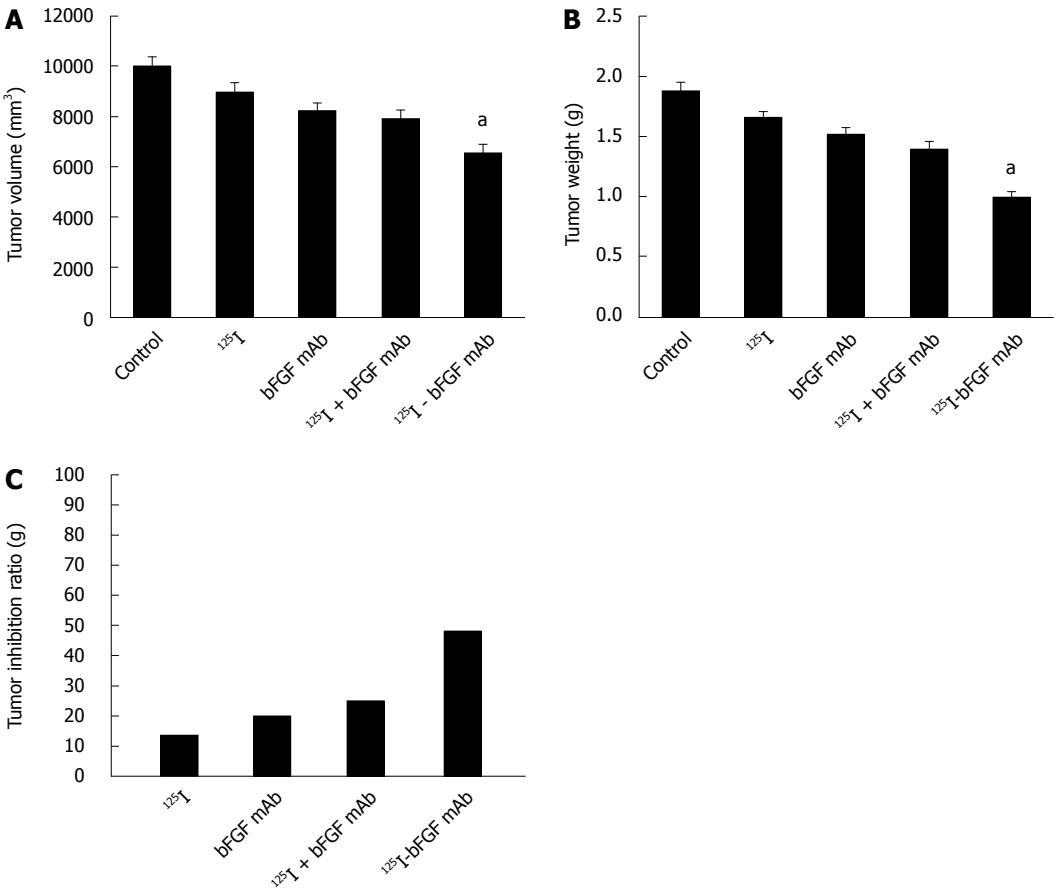
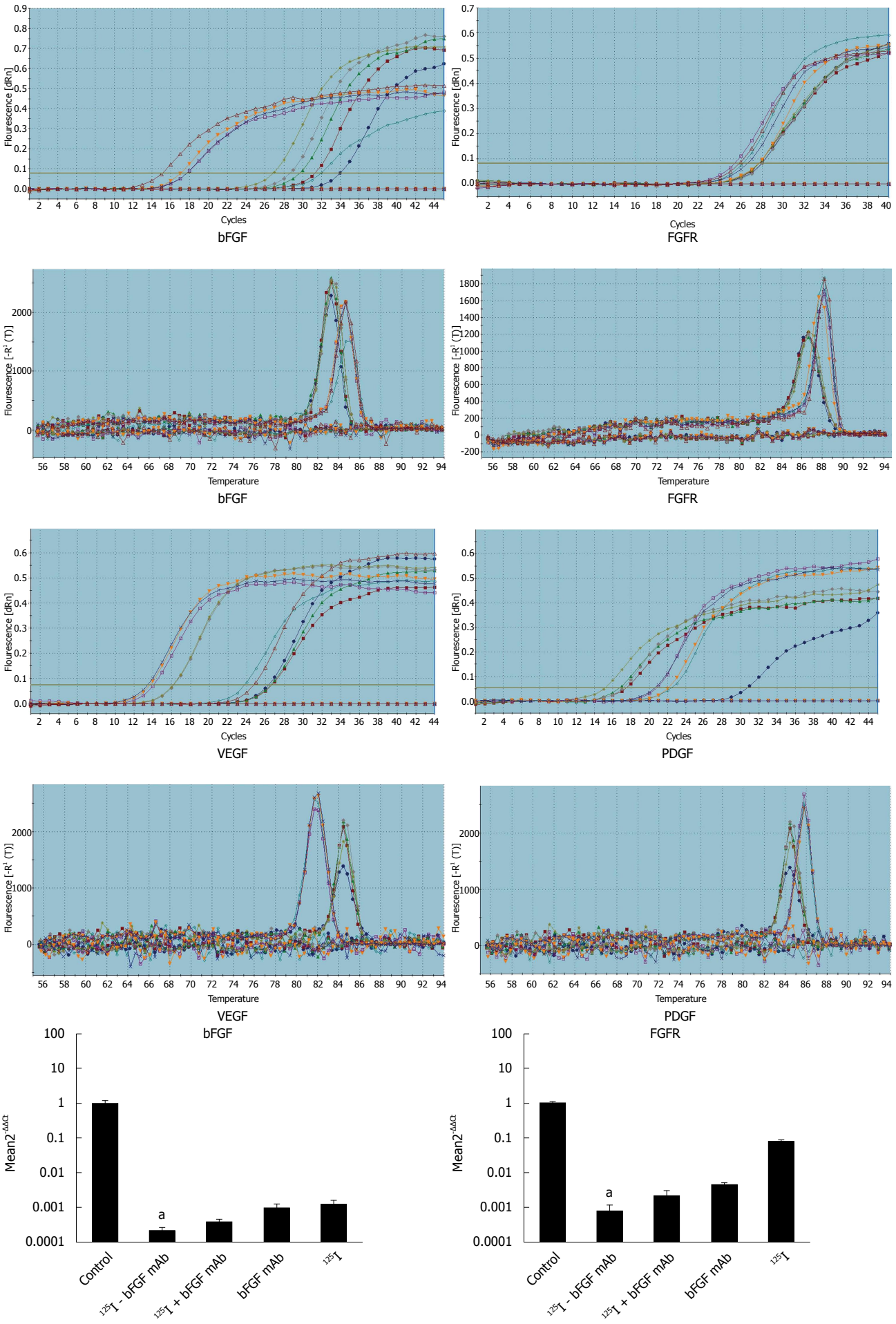


Figure 3 Therapeutic efficacy of ^{125}I-bFGF mAb in mice with H22 hepatocellular carcinoma. A: Tumor volume of H22 HCC; B: Tumor weight of H22 HCC; C: Tumor inhibition ratios of experimental groups; $^{a}P < 0.05$, ^{125}I-bFGF mAb group *vs* other groups (control, ^{125}I, bFGF mAb and ^{125}I plus bFGF mAb). HCC: Hepatocellular carcinoma.

amplification. All the samples were amplified with a single product, and there was no non-specific amplification. According to the relative quantitative method of $2^{-\Delta\Delta Ct}$, the relative expression of bFGF and FGFR mRNA decreased significantly in the ^{125}I-bFGF mAb group when compared with other treatment groups ($P < 0.05$). In groups with interventions, expression of PDGF mRNA decreased while VEGF mRNA was higher (except for ^{125}I group) than that in the control group (Figure 4).


Flourescence [dRn]
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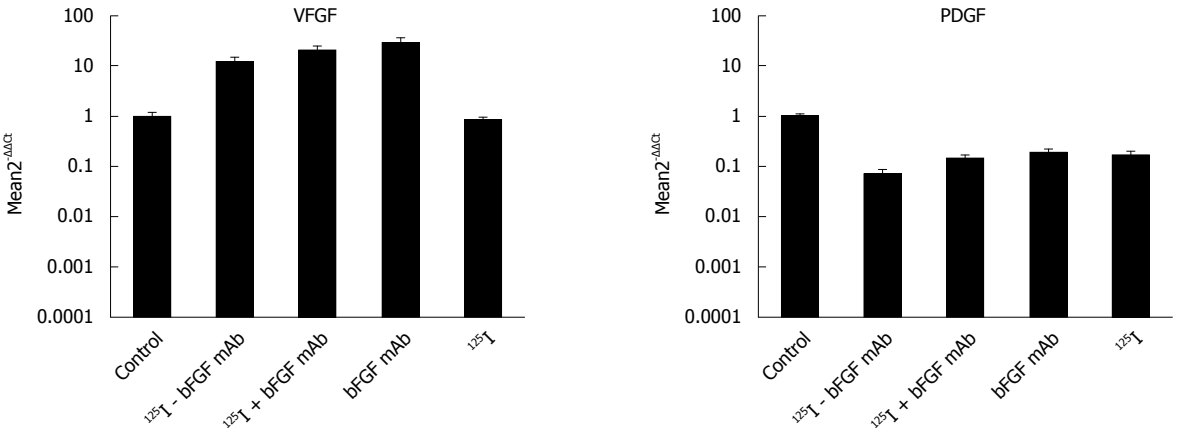

Figure 4 Quantitative real-time reverse transcriptase polymerase chain reaction amplification curves, melt curves and expression of bFGF, FGFR, VEGF and PDGF mRNA. $^{a}P < 0.05$, ^{125}I-bFGF mAb group *vs* other groups (control, ^{125}I, bFGF mAb and ^{125}I plus bFGF mAb). qRT-PCR: Quantitative real-time reverse transcriptase polymerase chain reaction.

DISCUSSION

bFGF is an 18 ku non-glycosylated polypeptide consisting of 146 amino acids that was first isolated and purified from bovine pituitary and brain. It is involved in cell migration and differentiation and is a driving force of mitogenesis and angiogenesis[5]. bFGF has been shown to disrupt the balance of cell cycle progression and apoptosis[16]. By activating protein kinase B, it enhances proliferation of HCC cells *via* the phosphoinositide 3-kinase pathway[17]. Growth of HCC was inhibited by human sulfatase 1, a bFGF-stimulated signaling blocker[16]. Moreover, it was previously demonstrated that a novel mAb to FGF-2 alone, without radiolabeling, effectively inhibited the growth of HCC xenografts[18].

Our results showed that ^{125}I-bFGF mAb significantly inhibited growth of HCC xenografts more than the other interventions ($P < 0.05$) and that the inhibition ratio of the ^{125}I-bFGF mAb group (47.9%) was higher than that of the ^{125}I plus bFGF mAb group (25.1%). Combining ^{125}I and bFGF mAb was more effective than concomitant use of ^{125}I and bFGF mAb in the treatment of HCC. The use of radionuclide labels on mAbs enhanced the specificity of cellular targeting[14]. Such augmented specificity and accuracy could allow ^{125}I-bFGF mAb to yield greater efficacy in treating mice with HCC compared with concomitant use of ^{125}I and bFGF mAb. Among patients with HCC, the serum levels of bFGF were increased, and elevated bFGF independently predicted poor disease-free survival preoperatively[8]. It is tempting to consider ^{125}I-bFGF mAb as a potential clinical option for HCC therapy in the future.

^{125}I-bFGF mAb reduced levels of FGFR and PDGF in our study. FGFR plays a pivotal role in HCC differentiation, proliferation, invasiveness, and resistance to chemotherapy[19-21]. FGFR is highly expressed in HCC and is associated with short overall survival[22]. A humanized monoclonal antibody to FGFR was reported to inhibit tumor growth in HCC xenograft models[23]. In contrast, PDGF, a proangiogenic factor, contributes to vessel maturation[24] and aids in the proliferation and metastasis of HCC[25]. Upregulation of PDGF and PDGF receptors is associated with chemoresistance of gemcitabine and poor prognosis in patients with HCC[26,27]. ^{125}I-bFGF mAb is also a promising agent in tackling liver cancer by decreasing both FGF and PDGF. Perhaps ^{125}I-bFGF mAb enhances therapeutic efficacy of gemcitabine when both agents are indicated in patients with HCC; this possibility requires further investigation.

Our results showed that the expression of VEGF was higher in mice treated with ^{125}I-bFGF mAb, ^{125}I plus bFGF mAb, and bFGF mAb in spite of the improved tumor inhibition ratios and decreased levels of bFGF, FGFR, and PDGF. Previously, increased expression of bFGF was observed in xenotransplanted squamous cell carcinoma after anti-VEGF treatment[28]. Antiangiogenic therapy may impair vessel formation but improve vascular function and tissue oxygenation[29]. Such vessel normalization may become a compensatory reaction of the tumor in response to the depletion of VEGF, leading to increased oxygenation and the observed increased bFGF[28]. In our study, application of anti-bFGF to a murine model of HCC increased VEGF, suggesting that blockade of VEGF elevates bFGF and vice versa. We speculate that vessel normalization also takes place even when anti-bFGF (an antiangiogenic agent) is used and that VEGF is increased by improved tissue oxygenation. Bevacizumab, a potent VEGF inhibitor A, was the first VEGF inhibitor approved by the United States Food and Drug Administration, and it demonstrates modest antitumor activity across a broad range of malignancies when combined with chemotherapy[30]. However, some patients are insensitive to bevacizumab. One study found that a VEGF/bFGF ratio correlated more closely with sensitivity to bevacizumab than with VEGF alone[31]. We found that ^{125}I-bFGF mAb increased

expression of VEGF in the HCC group. Therefore, we hypothesized that ^{125}I-bFGF mAb in combination with VEGF mAb may enhance sensitivity to bevacizumab and improve efficacy in the treatment of HCC. In the future, we will determine the effect of combination ^{125}I-bFGF mAb and bevacizumab on the treatment of HCC.

A recent study found using gefinitib-resistant cell lines that the expression of FGFR1 and bFGF was elevated and that inhibiting either bFGF or FGFR1 by small interfering RNA (siRNA) or FGFR inhibitor (PD173074) restored gefitinib sensitivity. These findings implicate activation of an FGFR autocrine loop as a mechanism of acquired resistance to epithelial growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) in non-small cell lung cancer[32]. Since ^{125}I-bFGF mAb can decrease significantly bFGF and FGFR, combining ^{125}I-bFGF mAb and EGFR-TKIs might enhance the therapeutic value of EGFR-TKIs.

In conclusion, ^{125}I-bFGF mAb effectively inhibited the growth of HCC xenografts; significantly reduced expression of bFGF and FGFR; and upregulated VEGF expression. Combined ^{125}I and bFGF mAb was more effective than concomitant use of ^{125}I and bFGF mAb in the treatment of HCC.

COMMENTS

Background

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide and the third leading cause of cancer-related death. In clinical practice, the majority of HCC patients are diagnosed at an inoperable stage, resulting in a low long-term survival rate and poor prognosis. Since basic fibroblast growth factor (bFGF) is one of the most prominent angiogenesis-related factors, angiogenesis plays an important role in HCC progression. Here, the authors investigated the biological inhibition efficacy of ^{125}I-labeled bFGF monoclonal antibody (mAb) in mice with HCC. In the near future, these findings might be helpful to clinicians selecting individualized treatment strategies.

Research frontiers

Targeted therapy is one of the main treatment approaches for patients with advanced HCC. New targeted drugs, such as sorafenib and sunitinib, have improved clinical efficacy. However, drug resistance and side effects of sorafenib and sunitinib constrain their clinical application. Therefore, it is necessary to investigate alternative targeting drugs, such as mAb to bFGF, for patients with advanced HCC.

Innovations and breakthroughs

To the best of our knowledge, this is the first study to label bFGF mAb with ^{125}I for the treatment of HCC. The study revealed that ^{125}I-bFGF mAb inhibited growth of HCC xenografts more effectively than the concomitant use of ^{125}I and bFGF mAb. The authors also found that ^{125}I-bFGF mAb reduced expression of bFGF, FGF receptor, and platelet-derived growth factor.

Applications

This study found that ^{125}I-bFGF mAb inhibited growth of HCC xenografts, suggesting that it could be used to tackle liver cancer. More trials are warranted to provide evidence for other applications. ^{125}I-bFGF mAb significantly inhibited the expression of bFGF and FGF receptor, while vascular endothelial growth factor (VEGF) expression was upregulated. Therefore, combination treatment of HCC with VEGF mAb is worthy of further investigation.

Terminology

bFGF mAb is a target drug that can specifically bind to bFGF and block its growth-stimulating activity. It is widely used in laboratory research, and it can significantly inhibit growth of human HCC cell lines *in vitro* and *in vivo*. Therefore, bFGF mAb could be a promising drug in the treatment of liver cancer.

Peer-review

This is a well-designed and executed project on the inhibitory efficacy of ^{125}I-bFGF mAb in HCC. The results show that ^{125}I-bFGF mAb inhibits growth of HCC xenografts more effectively than the concomitant use of ^{125}I and bFGF mAb. ^{125}I-bFGF mAb may be a potential clinical option for HCC therapy in the future.

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ORIGINAL ARTICLE

Basic Study

Transarterial administration of integrin inhibitor loaded nanoparticles combined with transarterial chemoembolization for treating hepatocellular carcinoma in a rat model

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Author contributions: Qian J designed the study and wrote the paper; Qian J, Oppermann E and Tran A performed the majority of experiments and analyzed the data; Qian K performed the nanoparticles preparation; Imlau U performed the molecular investigations; Vogl TJ coordinated the research and reviewed the paper.

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Abstract

AIM: To compare the effect of transarterial chemoembolization (TACE) plus GRGDSP (Gly-Arg-Gly-Asp-Ser-Pro, integrin-inhibitor) loaded nanoparticles with TACE alone or TACE + GRGDSP in a rat model of liver tumor.

METHODS: Morris hepatoma 3924A tumors were implanted in the livers of 30 ACI rats. The ACI rats were divided randomly into three groups (10 animals each). Tumor volume before treatment (V1) was examined by magnetic resonance imaging (MRI), and then, after laparotomy and placement of a PE-10 catheter into the hepatic artery, the following interventional protocols were performed: TACE (mitomycin C + lipiodol + degradable starch microspheres) + GRGDSP loaded nanoparticles for group A; TACE + GRGDSP

for group B (control group 1); TACE alone for group C (control group 2). Tumor volume (V2) was assessed by MRI and the mean ratio of the post-treatment to pretreatment tumor volumes (V2/V1) was calculated. Immunohistochemical analysis was performed to assess the quantification of matrix metalloprotein 9 (MMP-9) and vascular endothelial growth factor (VEGF) positive tumor cells in each treatment group.

RESULTS: The mean tumor growth ratios (V2/V1) were 1.3649 ± 0.1194 in group A, 2.0770 ± 0.1595 in group B, and 3.2148 ± 0.1075 in group C. Compared with groups B and C, group A showed a significant reduction in tumor volume. Lower expression of MMP-9 and VEGF in hepatocellular carcinoma was observed in group A than in groups B and C. The angiogenesis of tumor was evaluated using anti-VEGF antibodies, and the metastasis of tumor was assessed using anti-MMP-9 antibody. MMP-9 and VEGF were expressed in all specimens. The immunoexpression of these proteins was confirmed by the presence of red cytoplasmic staining in tumor cells. Lower expression of MMP-9 and VEGF in hepatocellular carcinoma was observed in group A than in groups B and C.

CONCLUSION: Transarterial administration of integrin inhibitor loaded nanoparticles combined with TACE evidently retards tumor growth and intrahepatic metastases compared with TACE alone or TACE plus integrin inhibitor in an animal model of hepatocellular carcinoma.

Key words: Hepatocellular carcinoma; Transarterial chemoembolization; Integrin inhibitor; Nanoparticles; Matrix metalloprotein 9; Vascular endothelial growth factor; ACI rats

Core tip: Our experimental study was designed to reduce tumor progression and recurrence through a combination of transarterial administration of GRGDSP (integrin-inhibitor) loaded nanoparticles plus transarterial chemoembolization (TACE) in an animal model of liver tumor. Our data showed that the combined biological and interventional treatment is a safe and effective therapy compared with TACE alone or TACE plus GRGDSP. The combined multimodal targeting therapies exhibit tremendous advantages over conventional interventional therapy alone.

Qian J, Oppermann E, Tran A, Imlau U, Qian K, Vogl TJ. Transarterial administration of integrin inhibitor loaded nanoparticles combined with transarterial chemoembolization for treating hepatocellular carcinoma in a rat model. *World J Gastroenterol* 2016; 22(21): 5042-5049 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i21/5042.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i21.5042

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignances worldwide and it has a poor prognosis due to its rapid infiltration, liver cirrhosis and metastases. Surgical resection and liver transplantation are regarded as potentially curative therapies for patients with HCC[1,2]. However, most patients are not suitable candidates for surgical approaches because of liver dysfunction, extrahepatic metastases, lack of donor organs and high recurrence rates. Currently, transarterial chemoembolization (TACE), percutaneous ethanol injection, radiofrequency ablation, microwave coagulation therapy, laser induced thermotherapy and cryotherapy are important components for minimally invasive therapy in patients with cirrhosis and unresectable primary or metastatic liver tumors[3-5]. TACE has been shown to reduce systemic toxicity and increase local effects and thus improve the therapeutic results[6]. However, the long-term survival rate of patients has not been substantiated in randomized clinical studies, mainly due to the tumor recurrence and metastases after treatment[5]. While it is well known that tumor metastasis is a multifactorial process, one key to tumor cell infiltration and metastasis is integrin-mediated adhesion of tumor cells to the normal basement membrane.

Integrin is a king of receptor molecules on the surface of cells, and the basic function of which is to mediate the intercellular adherence or adherence between cells and extracellular matrix (ECM). Integrin expressed by tumor cells and host cells can promote the progress of metastatic dissemination. Recently, studies of anti-integrin therapies are drawing more and more attention to the treatments that protect against recurrence and metastasis of tumors[7]. It was demonstrated that transarterial infusion of GRGDSP (Gly-Arg-Gly-Asp-Ser-Pro integrin-inhibitor which includes RGD-peptide) combined with TACE noticeably inhibited the growth of liver tumor in Wistar rats[8].

It is well known that the nanoparticles considered as drug carriers in the targeting treatment can change the drug distribution in the body, besides the benefit feature of slow drug release. Nanoparticles can be combined with different kinds of drugs or ligands for targeted drug delivery[9,10]. The nanoparticle-therapies have the potential to enhance the effect for inhibiting tumor proliferation and angiogenesis[11,12]. It was reported that the therapeutic effect of chemotherapeutic drug on liver tumor could be noticeably enhanced by the administration of nanoparticles *via* the hepatic artery. The rats that received Adriamycin loaded nanoparticles acquired obvious inhibition on tumor growth, as well as prolonged their survival[11,12]. However, to our knowledge, there have been no experimental or clinical reports on the therapeutic effectiveness of TACE combined with integrin inhibitor-loaded nanoparticles for treatment of HCC. Thus, the purpose of our study

was to assess the effect of TACE combined with GRGDSP loaded nanoparticles, compared with TACE alone or TACE plus GRGDSP for treating HCC in an animal model.

MATERIALS AND METHODS

Tumor cells and animal model

Morris hepatoma 3924A tumors, poorly differentiated HCC, was used in this study. The hepatoma cells were obtained from the German Cancer Research Center in Heidelberg. Thirty male ACI rats (200-220 g) were obtained from Harlan Winkelmann (Borchen, Germany). The experiments were performed in accordance with the German government and the institutional animal research review board. All the experiments were carried out under intraperitoneal anesthesia with ketamine hydrochloride (100 mg/kg), xylazin hydrochloride (15 mg/kg), and atropine sulfate (0.1 mg/kg).

Tumor implantation (day 0) was performed according to the method described by Yang *et al*[13] with slight modification[14]. The tumor tissue was recovered from an animal 12 d after subcutaneous implantation (5×10^6 tumor cells) and cut into small cubes (ca. 2 mm). The left lateral lobe of the liver of the recipient rat was exposed through a subxiphoid abdominal incision and a small subcapsular incision was made. The tumor fragment was gently embedded into the pocket and the abdominal wall was subsequently closed.

Agents

GRGDSP loaded nanoparticles were kindly provided by School of Life Science and Technology, Huazhong University of Science and Technology (Wuhan, China). GRGDSP loaded nanoparticles were synthesized using the method of Yang *et al*[15] with slight modifications. Superparamagnetic iron oxide (SPIO) was used as RGD (Arg-Gly-Asp) nanocarriers. The size and size distribution of the final product were determined by photon correlation spectroscopy (PCS) with a nano-ZS90 laser particle analyzer (Malvern Instruments Corp., United Kingdom). The mean diameter of particles was 107 nm, and the drug loading ratio was 50%.

A dose of 0.25 mg GRGDSP loaded nanoparticles was suspended in 0.6 mL OF 0.9% NaCl for 10 min before administration.

A dose of 0.1 mg mitomycin, 0.1 mL lipiodol and 5.0 mg degradable starch microspheres was administered into the hepatic artery of the rats in the experiment.

MR imaging (days 12 and 25)

One day before and 12 d after the interventional therapy, MRI was performed with a 3.0 Tesla Magnetom superconducting system (Siemens; Erlangen, Germany) using a wrist coil. MR images of the liver were acquired in the transverse plane using a T2-weighted turbo spin-echo sequence with the following imaging parameters: TR/TE, 3870/80 ms; slice thickness, 2 mm; matrix, 192 × 256. The tumor volume was evaluated in T2-weighted images according to the ellipsoid volume formula[16]: $V = 0.5 \times d1 \times d2^2$ (d1 = maximum diameter of the tumor; d2 = minimum diameter perpendicular to d1).

Interventional procedures (day 13)

A second laparotomy was performed 1 d after MRI examination for interventional treatment. A PE-10 polyethylene catheter (inner diameter 0.28 mm, outer diameter 0.61 mm, Wenzel, Heidelberg, Germany) was used for catheterization under a microscope. The catheter was inserted retrogradely into the gastroduodenal artery and pushed to the common hepatic artery. The following therapeutic agents were injected through the catheter to the hepatic artery by sandwich technique (sequential injection of lipiodol + mitomycin + GRGDSP loaded nanoparticles or GRGDSP + degradable starch microspheres):

Group A (TACE + GRGDSP loaded nanoparticles; n = 10): 0.1 mg mitomycin + 0.1 mL lipiodol + 5.0 mg degradable starch microspheres + 0.25 mg GRGDSP loaded nanoparticles.

Group B (control group 1, TACE + GRGDSP; n = 10): 0.1 mg mitomycin + 0.1 mL lipiodol + 5.0 mg degradable starch microspheres + 0.25 mg GRGDSP (2.5 mg/mL, Jingmei Biological, Wuhan, China).

Group C (control group 2, TACE alone; n = 10): 0.1 mg mitomycin + 0.1 mL lipiodol + 5.0 mg degradable starch microspheres.

Immunohistochemical examination (day 26)

All rats were sacrificed after the MRI examination by intravenous administration of overdose sodium pentobarbital. Liver samples were embedded and frozen in Tissue-Tek and 5 μm cryosections were generated. Sections were fixed in 100% acetone and endogenous peroxidase activity was blocked with 0.6% H_2O_2/MeOH followed by incubation with anti-MMP-9 rabbit polyclonal antibody (Cell Signaling Technology Inc., MA, United States) and/or anti-VEGF rabbit polyclonal antibody (Santa Cruz Biotechnology Inc., United States) overnight at 4 ℃. Sections were then incubated with an anti-rabbit alkaline phosphatase supervision polymer system (DCS Innovative Diagnostik-Systeme, Hamburg, Germany), and endogenous alkaline phosphatase was inhibited by 1 mmol/L levamisole present in the substrate. Sections were subsequently counterstained with hematoxylin and mounted in Kaisers Glycerol Gelatin (Merck, Darmstadt, Germany). To evaluate the expression of MMP-9 and VEGF, all slides were examined and scored by two independent pathologists who were blinded to the animal data. Stained cells were counted in 10 microscopic fields (× 100) per slide in tumor area and the average was calculated. Slides were evaluated in a semiquantitative method relating to the percentage staining of the cells and were scored as follows: 0 (No

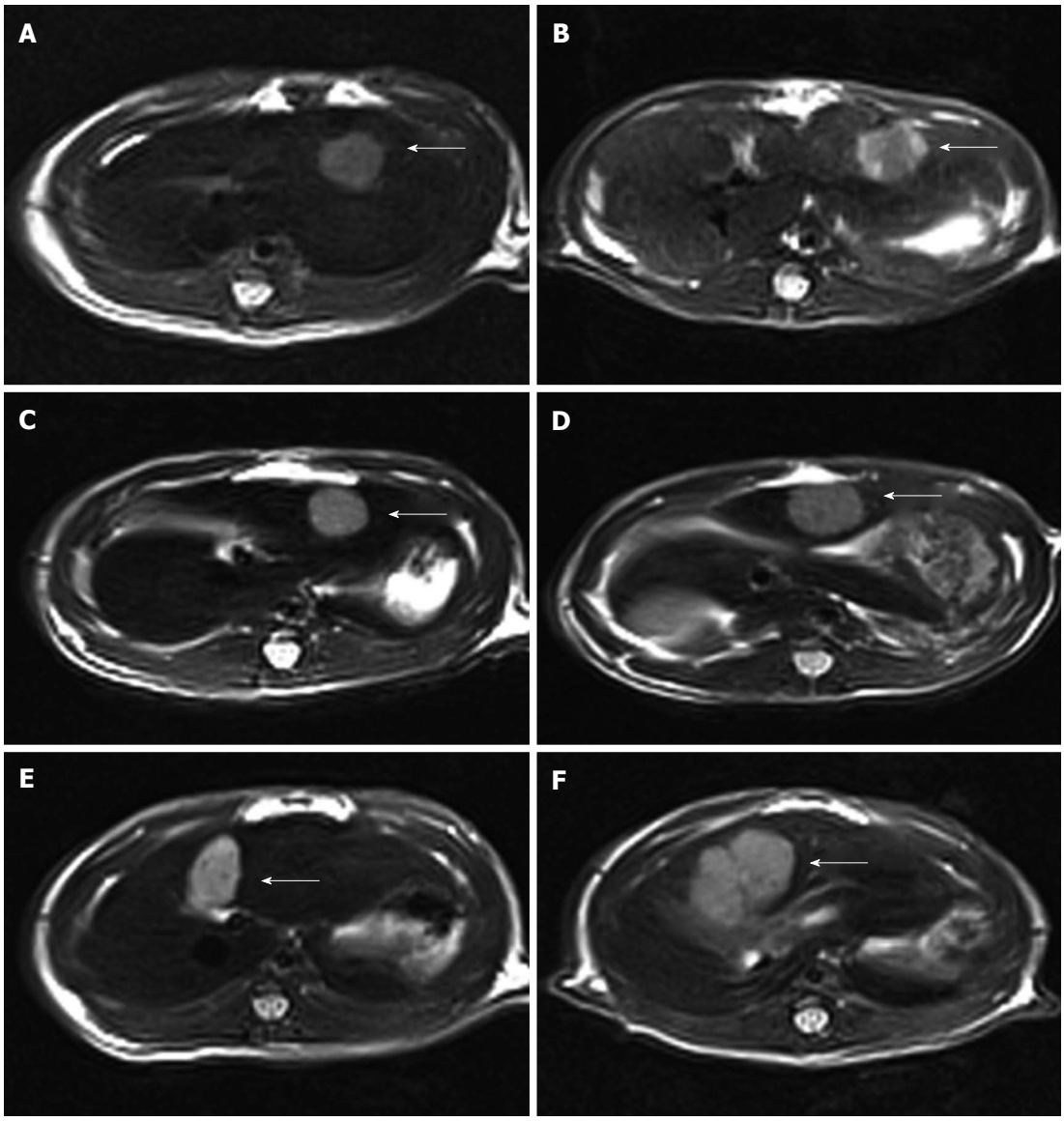

Figure 1 Representative transverse magnetic resonance images of solid liver tumors in group A (TACE+ GRGDSP loaded nanoparticles) (images A and B), group B (Control group 1, TACE+ GRGDSP) (images C and D) and group C (Control group 2, TACE alone) (images E and F) in ACI rats. 3870/80 matrix was acquired for all images. A: The pretreatment unenhanced T2-weighted TSE MR image shows a small hyperintense tumor (arrow) in the left lateral liver lobe (0.81 cm × 0.79 cm); B: On the posttreatment unenhanced T2-weighted TSE MR image, the same lesion (arrow) (0.85 cm × 0.82 cm) is a hyperintense tumor and has the inhomogeneous hypointense area corresponding to intratumoral necrosis. The growth of the hepatic tumor is noticeably inhibited after therapy; C: The pretreatment unenhanced T2-weighted TSE MR image shows a small hyperintense tumor (arrow) in the left lateral liver lobe (0.75 cm × 0.70 cm); D: On the posttreatment unenhanced T2-weighted TSE MR image, the same lesion (arrow) (1.03 cm × 0.87 cm) is a hyperintense tumor and has the inhomogeneous hypointense area corresponding to intratumoral necrosis. The hepatic tumor appears to have grown slightly after therapy; E: The pretreatment unenhanced T2-weighted TSE MR image shows a small hyperintense tumor (arrow) in the left lateral liver lobe (0.74 cm × 0.71 cm); F: On the posttreatment unenhanced T2-weighted TSE MR image, the same lesion appears as a 1.23 cm × 1.01 cm tumor (arrow) with relatively rapid growth compared to its size before therapy. TACE: Transarterial chemoembolization.

staining); 1 (0%-5%); 2 (6%-25%); 3 (26%-50%); 4 (51%-75%); and 5 (76%-100%).

Statistical analysis

The mean tumor growth ratio (V_2/V_1) by MRI from each group and the significance of differences were analyzed using the statistical software Prism (version 3.02, La Jolla, CA, United States).

Immunohistochemical staining of MMP-9 and VEGF was evaluated using descriptive and semiquantitative methods. Statistical analyses were performed using Prism (version 3.02, La Jolla, CA, United States).

Comparisons between groups were made using the Bonferroni test. Differences with a *P*-value less than 0.05 were considered statistically significant.

RESULTS

MRI examination

Tumor implantation was successful in all of the rats. Most tumors appeared homogeneous and were hypointense on T1-weighted images and hyperintense on T2-weighted images prior to treatment, but inhomogeneous after treatment. The mean growth

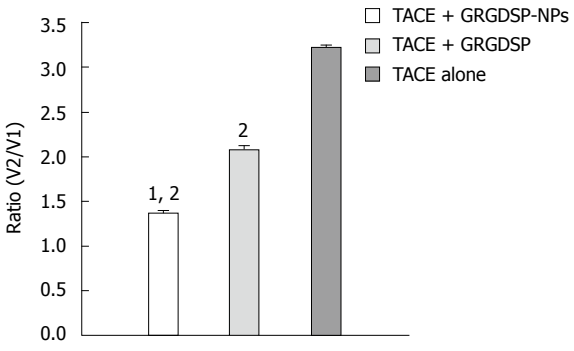

Figure 2 The mean tumor growth ratio (V_2/V_1) by magnetic resonance imaging from each group. The mean tumor growth ratio (V_2/V_1) by MRI showed significant differences between group A (TACE + GRGDSP loaded nanoparticles), group B (control group 1, TACE + GRGDSP) and group C (control group 2, TACE alone) ($P < 0.01$). TACE: Transarterial chemoembolization. [1]Compared with group B (control group 1, TACE + GRGDSP); [2]Compared with group C (control group 2, TACE alone).

ratios of tumors [V_2 (postreatment)/V_1 (pretreatment)] were 1.3649 ± 0.1194 in group A, 2.0770 ± 0.1595 in group B, and 3.2148 + 0.1075 in group C. Compared to groups B and C, group A (TACE + GRGDSP loaded nanoparticles) showed a significant reduction of tumor growth ($P < 0.01$) in the period of observation by Bonferroni test (Figures 1 and 2).

Immunohistochemical assay

The angiogenesis of tumor was evaluated using anti-VEGF antibody, and the metastasis of tumor was assessed using anti-MMP-9 antibody. MMP-9 and VEGF were expressed in all specimens. The immunoexpression of these proteins was confirmed by the presence of red cytoplasmic staining in tumor cells (Figures 3 and 4). Lower expression of MMP-9 and VEGF in HCC were observed in the group A than in groups B and C (controls) ($P < 0.01$) (Tables 1 and 2).

DISCUSSION

HCC is one of the most common malignancies with very high morbidity and mortality. TACE is a widely used palliative treatment for patients with unresectable HCC[4]. However, it has not led to significant improvements in the long-term survival rates, because of postoperative metastasis and recurrence of tumors[8]. Local infiltration and metastasis of tumors are a complicated process which is influenced by many factors.

The mechanism of adhesion molecules was reported to play an important role in the regulation of cellular migration, proliferation and apoptosis[17,18]. Integrin receptors are abnormally expressed on the surface of tumor cells, where they perform the basic function of mediating intercellular and cell-extracellular matrix (ECM) adherence. The adhesive function of integrins works by identifying the specific RGD sequence in the ligand (one part of ECM) and the links to it. RGD peptide is a kind of extrinsic peptide, which can competitively bind to integrin and inhibit binding with the RGD sequence of the ECM. The integrin-mediated adherence between tumor cells and ECM can be decreased by RGD peptide, and the inhibitory action is dose dependent. Furthermore, degradation of ECM caused by MMP-9 can be inhibited. Binding of RGD to integrin receptor $\alpha v\beta 3$, which is abnormally expressed in the endothelial cells of tumor blood vessels, may prevent blood vessel formation and infiltrating[17,19-21]. Therefore, RGD peptide can be regarded as a broad-spectrum antagonist of integrin. As a synthetic linear RGD peptide, GRGDSP (Gly-Arg-Gly-Asp-Ser-Pro) could inhibit the adherence of tumor cells to endothelial cells of blood vessels and limit its metastasis[22-24]. Tsuchiya *et al*[20] have found that intravenous administration of synthetic RGD pseudo-peptide (FC-336) could inhibit intrahepatic metastasis compared with control group ($P < 0.05$). Typically, a previous study has demonstrated that transarterial infusion of GRGDSP combined with TACE noticeably inhibited the growth of hepatic carcinoma and intrahepatic metastasis in Walker-256 rats[8]. Recently, nanobiotechnology has many advantages for improving drug delivery by the following approaches[10]. First, particle size can be reduced to nanometer size range to increase the surface area, thereby increasing the rate of dissolution. Second, nanoparticles can improve the absorption of insoluble compounds and macromolecules, enhance the bioavailability and release rates, and therefore reduce the amount of dose required and side effects. Finally, nanoparticles can be combined with ligands for targeted drug delivery. Nanotechnology is particularly useful for delivery of biological therapies. Nanotechnology will enable design and delivery of more effective drugs with increased efficacy and reduced toxicity. Wang *et al*[25] have showed that the nanoparticles coupled with RGD-peptide and doxorubicin represent high efficacy in inducing apoptosis in specific malignant cancer cells. Iwasaki *et al*[9] demonstrated that nanoparticles can be intravenously administrated for delivery of therapeutic genes with anti-tumor activity into human liver tumors. It was also reported that the therapeutic effect of adriamycin on liver malignancy can be significantly enhanced by its nanoparticle formulation and administration *via* the hepatic artery[11,12]. However, up to date no study has reported on the therapeutic effect of nanoparticles combined with TACE for treating HCC *in vivo* or in clinic. Thus, our experimental study was designed to reduce tumor progression and recurrence by combination of transarterial administration of GRGDSP loaded nanoparticles with TACE by using sandwich technique in an animal model of HCC. Our experimental results showed that transarterial administration of GRGDSP loaded nanoparticles + TACE can significantly inhibit the growth of hepatic tumor and intrahepatic metastases. Lower expression of MMP-9 and VEGF in HCC was observed in the group A (TACE + GRGDSP loaded-nanoparticles) than in group

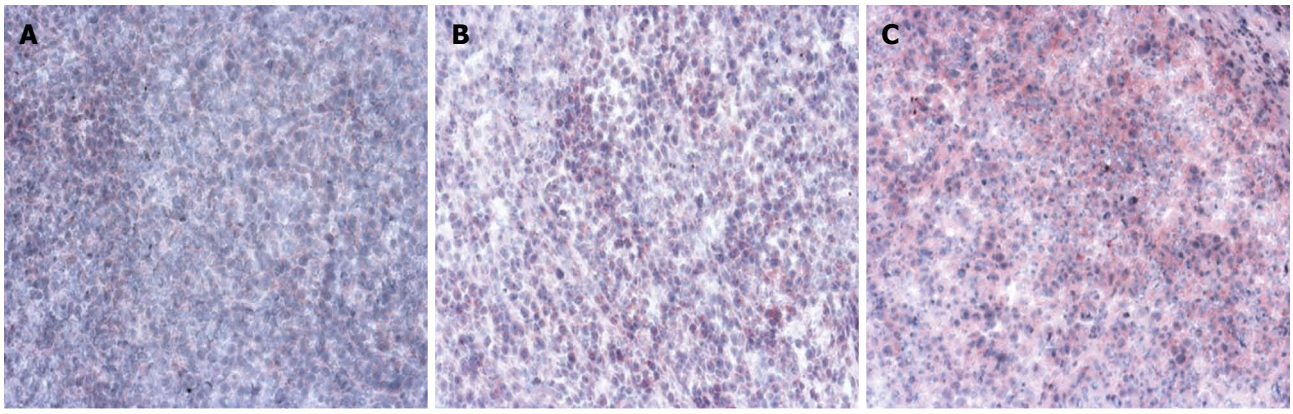

Figure 3 Immunohistochemical staining of VEGF in hepatocellular carcinoma in group A (TACE + GRGDSP loaded nanoparticles), group B (Control group 1, TACE + GRGDSP) and group C (Control group 2, TACE alone) (magnification × 100). A: VEGF expression in hepatocellular carcinoma in group A. B: Higher expression of VEGF in hepatocellular carcinoma was observed in group B than in group A; C: Higher immunohistochemical expression of VEGF in hepatocellular carcinoma was observed in group C than in groups A and B. VEGF: Vascular endothelial growth factor; TACE: Transarterial chemoembolization.

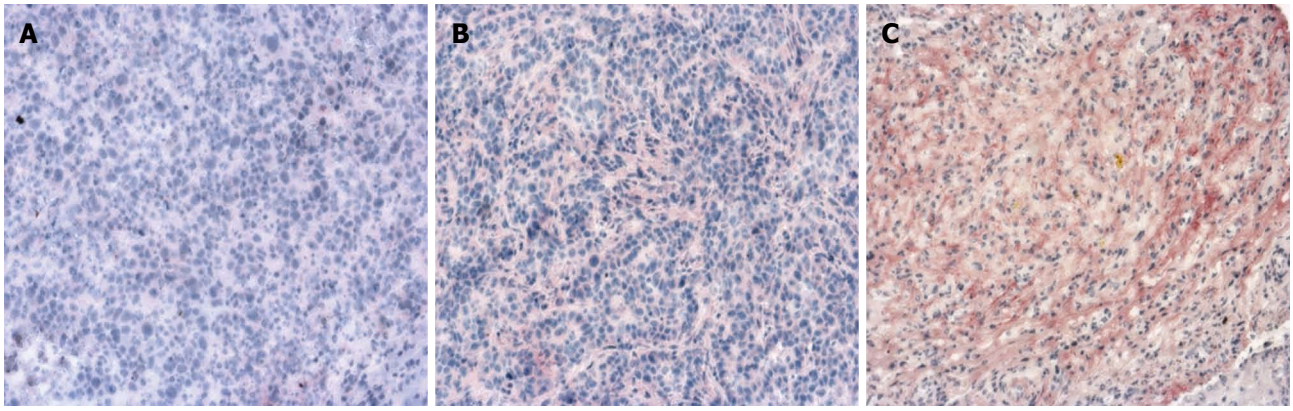

Figure 4 Immunohistochemical staining of matrix metalloprotein 9 in hepatocellular carcinoma in group A (TACE + GRGDSP loaded nanoparticles), group B (Control group 1, TACE + GRGDSP) and group C (Control group 2, TACE alone) (magnification × 100). A: Immunohistochemical staining of MMP-9 in hepatocellular carcinoma in group A. B: Higher immunohistochemical expression of MMP-9 in hepatocellular carcinoma was observed in group B than in group A. C: Higher immunohistochemical expression of MMP-9 in hepatocellular carcinoma was observed in group C than in groups A and B. MMP-9: Matrix metalloprotein 9; TACE: Transarterial chemoembolization.

Table 1 Immunohistochemical expression of matrix metalloprotein 9 and vascular endothelial growth factor in hepatocellular carcinoma (%) in groups A and B

	Group	TACE + GRGDSP						TACE + GRGDSP loaded nanoparticles						*P* value
	Score	0	1	2	3	4	5	0	1	2	3	4	5	
VEGF	Tumor	3	13	28	20	20	16	29	5	18	20	18	10	0.000
MMP-9	Tumor	0	1	17	18	42	22	0	6	36	41	12	5	0.000

MMP-9: Matrix metalloprotein 9; VEGF: Vascular endothelial growth factor; TACE: Transarterial chemoembolization.

B (TACE + GRGDSP) and group C (TACE alone). The invasive progression and metastases of tumor cells in group A were noticeably inhibited compared with the control groups.

For application in TACE, lipiodol not only occludes the small arteries supplying the tumor, but can also be used as the carriers bringing the anticancer drugs to the tumor. Lipiodol can deliver cytotoxic agents directly into tumor cells and endothelial cells, enter into the microcirculation of the tumor and block the blood flow[26-28]. Anticancer drugs administered through TACE can escape first-pass metabolism and have a prolonged half-life[29]. Moreover, the currently synthesized GRGDSP loaded nanoparticles have a mean diameter of 107 nm. It was documented that the passive targeting ability of the nanoparticles depends on the vessel microstructures of target organs. The nanoparticles with a diameter ranging from 20 to 300 nm have also the ability to directly enter the hepatocytes[30].

Table 2 Immunohistochemical expression of matrix metalloprotein 9 and vascular endothelial growth factor in hepatocellular carcinoma (%) in groups A and C

	Group	TACE						TACE + GRGDSP loaded nanoparticles						*P* value
	Score	0	1	2	3	4	5	0	1	2	3	4	5	
VEGF	Tumor	0	2	10	11	16	61	29	5	18	20	18	10	0.000
MMP-9	Tumor	0	6	4	0	9	81	0	6	36	41	12	5	0.000

MMP-9: Matrix metalloprotein 9; VEGF: Vascular endothelial growth factor; TACE: Transarterial chemoembolization.

In conclusion, encouraging results were obtained by combining transarterial administration of integrin inhibitor loaded nanoparticles with TACE for treating HCC in rats in comparison with control groups, and may prove valuable to human application as a therapeutic approach for the treatment of HCC. The combined multimodal targeting therapies reveal their enormous advantages as compared with conventional interventional therapy alone. However, detailed therapeutic mechanisms, therapeutic indications, monitoring and side effects of these combined therapies remain unclear and require more randomized experimental studies.

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COMMENTS

Background

Transarterial chemoembolization (TACE) was introduced as one of the most common forms of interventional therapy but its therapeutic effect combined with gene therapy remains to be elucidated.

Research frontiers

Hepatocellular carcinoma (HCC) is one of the most commonly occurring tumors worldwide and TACE was introduced as an effective treatment in patients with unresectable HCC. Integrins expressed by tumor cells and host cells can contribute directly to the control and progress of metastatic dissemination. The authors have previously demonstrated the encouraging results of interventional therapy of TACE plus GRGDSP compared with TACE or GRGDSP alone. Transarterial infusion of integrin inhibitor (GRGDSP) loaded nanoparticles plus TACE may be a safe and effective therapy targeting metastatic dissemination of tumor cells.

Innovations and breakthroughs

This study for the first time evaluates the effects of TACE plus GRGDSP loaded nanoparticles compared with TACE alone or TACE plus GRGDSP for treating HCC in an animal model. Its results indicate that transarterial administration of GRGDSP loaded nanoparticles combined with TACE evidently retards tumor growth and intrahepatic metastases compared with TACE alone or TACE plus GRGDSP in rats.

Applications

Integrin inhibitor loaded nanoparticles combined with TACE might be used as a new therapeutic approach for the treatment of HCC and inhibition of intrahepatic metastasis after TACE.

Terminology

Integrin is a receptor molecule on the surface of cells, and its basic function is to mediate the intercellular adherence or adherence between cells and extracellular matrix. GRGDSP (Gly-Arg-Gly-Asp-Ser-Pro, integrin-inhibitor) can prevent the adhesion of tumor cells and endothelial cells of blood vessels, and also inhibit the metastasis of tumor cells.

Peer-review

The manuscript investigated the effect of combined administration of TACE and GRGDSP-conjugated nanoparticles in a rat model of HCC. The study appears to be well performed and the manuscript is well written. This study is more a pilot study rather than an exhaustive study, but is worth publishing. Subsequent studies should be performed to localize the nanoparticles shortly after the administration and to investigate the distribution of the nanoparticles in the liver.

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ORIGINAL ARTICLE

Case Control Study

Danish cohort of monozygotic inflammatory bowel disease twins: Clinical characteristics and inflammatory activity

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Author contributions: Moller FT, Knudsen L, Harbord M, Satsangi J, Gordon H, Christiansen L, Christensen K, Jess T and Andersen V concepted and designed the study; Moller FT performed generation and analysis of data; Moller FT, Jess T and Andersen V interpreted the data; Moller FT drafted the manuscript; Moller FT, Andersen V, Jess T, Knudsen L, Harbord M, Satsangi J, Gordon H, Christiansen L and Christensen K performed critical revision and approved of the final version of the manuscript.

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Institutional review board statement: The study was approved by the ethics committee of the region of southern Denmark (approval No: S20120176). Further, the study is included in the regional application to The Data Protection Agency (Institutional Southern Region of Denmark J.nr. 2008-58-0035). To ensure confidentiality direct paired comparisons between twin pairs are not shown.

Informed consent statement: Verbal as well as written informed consent was obtained from participants. This included consent to contact co-twins of the index twins, even if that included informing the co-twin of the diagnosis of the index twin.

Conflict-of-interest statement: Vibeke Andersen is an adviser for MSD/Merck, Jansen, and member of advisory board for MSD/Merck; Tine Jess has received funding for travel and speakers fee from AbbVie; Frederik Trier Moller, Lina Knudsen, Marcus Harbord, Jack Satsangi, Hannah Gordon, Lene Christiansen, Kaare Christensen: no conflicts of interest.

Data sharing statement: Technical appendix, code is available from the corresponding author at (frtm@ssi.dk). Additional data are available on request but may require further IRB approval/approvals from the data protection agency, to be shared outside the research group.

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Abstract

AIM: To describe the establishment of a Danish inflammatory bowel diseases (IBD) twin cohort with focus on concordance of treatment and inflammatory markers.

METHODS: We identified MZ twins, likely to be discordant or concordant for IBD, by merging information from the Danish Twin Register and the National Patient Register. The twins were asked to provide biological samples, questionnaires, and data access to patient files and public registries. Biological samples were collected *via* a mobile laboratory, which allowed for immediate centrifugation, fractionation, and storage of samples. The mean time from collection of samples to storage in the -80 ℃ mobile freezer was less than one hour. The diagnoses where validated using the Copenhagen diagnostic criteria.

RESULTS: We identified 159 MZ IBD twin pairs, in a total of 62 (39%) pairs both twins agreed to participate. Of the supposed 62 IBD pairs, the IBD diagnosis could be confirmed in 54 pairs. The cohort included 10 concordant pairs, whereof some were discordant for either treatment or surgery. The 10 concordant pairs, where both pairs suffered from IBD, included eight CD/CD pairs, one UC/UC pair and one UC/IBDU pair. The discordant pairs comprised 31 UC, 5 IBDU (IBD unclassified), and 8 CD discordant pairs. In the co-twins not affected by IBD, calprotectin was above 100 μg/g in 2 participants, and above 50 μg/g in a further 5 participants.

CONCLUSION: The presented IBD twin cohorts are an excellent resource for bioinformatics studies with proper adjustment for disease-associated exposures including medication and inflammatory activity in the co-twins.

Key words: Digestive system diseases; Inflammatory bowel diseases; Crohn's disease; Ulcerative colitis; Epidemiologic studies; Twins; Biobank

Core tip: Using co-twin study designs to segregate genetic and environmental factors in inflammatory bowel diseases (IBD) holds promise for future discovery, considering subclinical disease in the co-twins. However, as MZ IBD discordant twins are rarely seen this often-mean insufficient power for planned analyses. Hence, collaboration between IBD twin resources is crucial.

Moller FT, Knudsen L, Harbord M, Satsangi J, Gordon H, Christiansen L, Christensen K, Jess T, Andersen V. Danish cohort of monozygotic inflammatory bowel disease twins: Clinical characteristics and inflammatory activity. *World J Gastroenterol* 2016; 22(21): 5050-5059 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i21/5050.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i21.5050

INTRODUCTION

The inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC), affect a large number of Europeans[1,2]. Despite the introduction of new treatments, CD and UC remain chronic conditions with severe disease morbidity, often complicated by surgery and frequent admissions to hospital[1,3].

Although studies of the genome have found 200 loci associated with IBD, the variation in the IBD phenotype explained by these finding is still below 25%-30%[4,5] suggesting a role of environmental factors in IBD pathogenesis. Several studies indicate environmental impact on IBD pathogenesis including; exposure to pathogens[6], disease associated dysbiosis[7], metabolic disequilibrium[8], or epigenetic modifications[9]. More comprehensive studies, addressing these and other potential causes of IBD, could provide invaluable new insight into the pathogenesis of IBD[10], though studies using unrelated individuals would require large populations to overcome genetic variation between unrelated subjects[11].

Monozygotic (MZ) twins share common genotypes and epigenetic profiles at conception[12]. While some epigenetic differences arise during the lifetime of MZ twins[13], the inter-individual variation in relation to *e.g.,* the epigenome and the gut microbiome remain lower between twin pairs than between unrelated persons[14]. Consequently, comprehensive studies of the exposome using IBD discordant MZ twin study designs could prove a powerful tool to assess the combined effects of environmental and endogenous factors, and identify targets for treatment and prevention[15].

A major challenge in such discordant twin pair studies is that quiescent or subclinical disease may blur the boundaries between cases and their co-twin controls[16,17]. Another major challenge is that IBD discordant twin pairs are also treatment discordant, hence observed differences might derive from differential medication rather than disease discordance. Given enough power, studies using concordant twin pairs in addition to discordant twin pairs could allow researchers to adjust for disease-associated exposures such as medication, as both twins have IBD but may be discordant for some of the applied treatments. Further, calprotectin correlates with intestinal inflam-

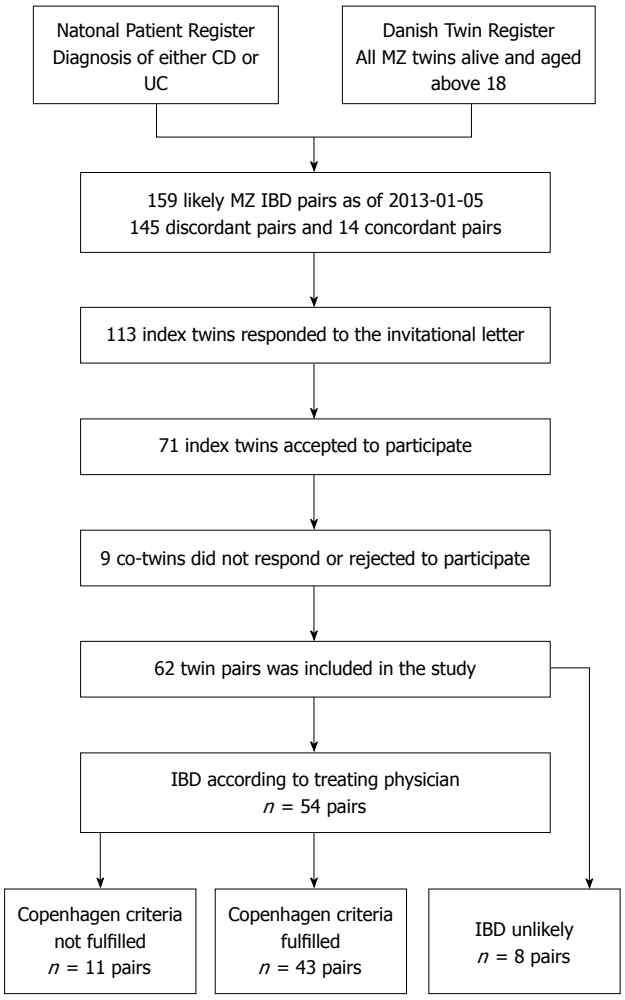

Figure 1 Collection of twin pairs. IBD: Inflammatory bowel diseases; CD: Crohn's disease; UC: Ulcerative colitis.

mation[18-20], and could reflect quiescent or subclinical disease in the unaffected co-twins.

We describe the establishment of a Danish IBD twin cohort including sampling of biological material, and illustrate the importance of treatment discordance and measurement of inflammatory markers for future bioinformatics studies using IBD affected twins.

MATERIALS AND METHODS

We identified MZ twins, likely to be discordant or concordant for IBD, by merging information from the Danish Twin Register and the National Patient Register[21,22].

Danish twin register

The Danish Twin Register enabled identification of MZ twins living in Denmark at the time of inclusion, with assessment of zygosity correct in 96% of cases[23]. The Register contains 72% of all twin pairs born between 1931-1968, with complete ascertainment of all live born twins since 1968[21].

National patient register

The National Patient Register is a nationwide register of all hospital discharge diagnoses, including surgical and other procedures recorded in Danish hospitals since 1977[22]. The Register provides outpatient data from 1994 and surgical procedures since 1996. Diagnoses of UC and CD were identified using the international classification of diseases (ICD) 8th and 10th revision codes for CD (563.00-563.09 and K50) and UC (563.19, 569.04 and K51).

The diagnosis of IBD has previously been found to be accurate in over 90% of IBD cases in the national patient register, using a pathology register as reference[24].

Cohort recruitment

Merging the Danish Twin Register and the National Patient Register, identified 159 MZ twin pairs in which at least one twin had a diagnosis of either CD or UC according to the National Patient Register as of May 1st 2013. Of these, 113 index twins (the first twin to contract IBD according to the register) responded to the invitational letter of whom 42 twins declined to participate. Of the 71 positive index twin responders, nine co-twins did not wish to participate, leaving 62 pairs for inclusion, Figure 1.

Data collection

The participants filled out a questionnaire including age, sex, smoking status, medication, dietary patterns including a food frequency questionnaire, a 48-h dietary recall, time of last meal or exercise, travel history, and pregnancies and disease activity at time of sampling, either Harvey Bradshaw Index (CD) (33) or Simple Clinical Colitis Index (UC) (34).

Data collected from the patient record included disease staging using the Montreal classification (32), any IBD complications, extra intestinal manifestations, and gastrointestinal operations as well as prior IBD medication.

The register diagnosis of CD, UC or IBDU, was validated by hospital records and pathology descriptions using the Copenhagen criteria[25]: Copenhagen Diagnostic Criteria for CD (at least two of the criteria present)[26,27]: (1) History of abdominal pain, weight loss and/or diarrhoea for more than three months; (2) Characteristic endoscopic findings of ulceration (aphthous lesions, snail track ulceration) or cobble stoning or radiological features of stricture or cobble stoning; (3) Histopathology consistent with Crohn's disease (epitheloid granuloma of Langerhans type or transmural discontinuous focal or patchy inflammation); and (4) Fistula and/or abscess in relation to affected bowel segments.

Copenhagen diagnostic criteria for UC (all three of the criteria present)[26,28]: (1) History of diarrhoea and/

Table 1 Clinical characteristics *n* (%)

Pair type	Discordant twin pairs					Concordant twin pairs	
Status	Co-twin	CD	IBDU	UC	non-IBD GI symptoms	IBD co-twin	IBD index twin
n	52	8	5	31	8	10	10
Males/Females	23/29	2/6	1/4	16/15	4/4	4/6	4/6
Age (yr)	50(26-78)	47 (26-67)	57 (34-77)	49 (32-70)	55 (27-78)	49 (28-68)	49 (28-68)
Age at onset		32 (21-47)	43 (23-73)	35 (20-59)	38 (23-62)	21 (14-29)	23 (11-34)
Age at diagnosis		34 (25-46)	41 (29-72)	34 (17-66)	48 (18-72)	24 (11-37)	31 (21-47)
CPH criteria fulfilled		6 (75)	4 (80)	24 (77)	0 (0)	8 (80)	9 (90)
Disease location							
L1 ileal		2 (25)	2 (40)			3 (30)	2 (20)
L2 colonic		3 (38)	0 (0)			1 (10)	1 (10)
L3 ileocolonic		0 (0)	0 (0)			4 (40)	4 (40)
L4 isolated upper disease		0 (0)	0 (0)			0 (0)	1 (10)
B1 non stricturing non penetrating		4 (50)	2 (40)			3 (30)	1 (10)
B2 stricturing		2 (25)	0 (0)			4 (40)	5 (50)
B3 penetrating		0 (0)	0 (0)			3 (30)	2 (20)
P perianal disease		1 (13)	0 (0)			2 (20)	0 (0)
Proctitis			1 (20)	6 (19)			
Left sided			0 (0)	9 (29)			
Extensive			1 (20)	10 (32)			

n denotes the number of participants with the phenotype described in status. IBD: Inflammatory bowel diseases; CD: Crohn's disease; UC: Ulcerative colitis.

or rectal bleeding and pus for more than one week or repeated episodes; (2) characteristic endoscopic findings of continuous ulceration, vulnerability or granulated mucosa; and (3) histopathology consistent with ulcerative colitis (neutrophils within epithelial structures, cryptitis, crypt distortion, crypt abscesses).

Inter-observer variation has previously been found with regards to the Montreal classification[29]. To avoid potential inter-observer variation one researcher validated the diagnoses and assessed the Montreal classification (FTM). Furthermore, to improve validity of diagnoses and phenotypes, complicated cases were reviewed by a gastroenterological specialist and senior physician (VAN). In daily clinical practice, the diagnosis may remain difficult; therefore, we included cases, which were perceived to have IBD by the treating physician although not fulfilling the Copenhagen criteria as IBD cases according to available information from the files. Cases where the diagnosis of IBD was unlikely were designated "Gastrointestinal (GI) symptoms not IBD" for future reference.

Biological samples

Due to the geographical challenges in sampling a nationwide cohort, a mobile lab was set up using a camper previously fitted for a similar purpose. The camper was equipped with a small lab bench, heating, refrigeration, -20 ℃ freezer, a mobile -80 ℃ freezer, as well as a swinging bucket centrifuge.

The mobile lab setup allowed researchers to visit the twins in their home or another private location. The samples were collected adhering to the Sample PRE-analytical Code (SPREC) and Biospecimen Reporting for Improved Study Quality (BRISQ) guidelines, logging primary container, pre- and post-centrifugation conditions, centrifugation parameters and storage conditions, see supplementary materials Table 1[30,31]. Faecal specimens were sampled by participants up until 48 h before the visit and stored in their own freezer at -20 ℃[32]. Samples were then transferred to a -80 ℃ freezer at the visit, under which conditions faecal samples have been found to be stable in composition[33]. Oral samples were collected with a cytobrush (Cytotak™ Transwab® Labelled Tube MW148) from the dorsum of the tongue, suspended in a buffer medium, and immediately frozen at -80 ℃. Paraffin was used to collect sputum samples that were either suspended in RNA later or frozen directly at -80 ℃. One researcher conducted the collection of all samples. All samples were analysed using standard methods centrally to avoid sampling variation between different centers.

The mean time from collection of samples to storage in the -80 ℃ mobile freezer was less than one hour, except for blood samples, which were 60 min and 15 s please see Supplementary materials Table 2. Records where kept to ensure identification of any deviations from protocol in future analysis.

Statistical analysis

The study included only basic descriptive statistics using R version 3.2.0. In order to ensure confidentiality, no grouping of the twins below five pairs was presented. The statistical methods of this study were reviewed by statistician Mikael Andersson from department of epidemiology at Statens Serum Institut.

RESULTS

Study cohort

Out of 62 MZ twin pairs, after scrutinizing patient records, register data, and questionnaires, we found the index case of eight pairs unlikely to have IBD.

Table 2 Complications, medication and smoking *n* (%)

Pair type	Discordant twin pairs					Concordant twin pairs	
Status	Co-twin	CD	IBDU	UC	non-IBD GI symptoms	IBD co-twin	IBD index twin
n	52	8	5	31	8	10	10
Complications							
GI complications[1]	3 (6)	2 (25)	1 (20)	2 (6)	0 (0)	4 (40)	5 (50)
Extra intestinal manifestations[2]	5 (10)	3 (38)	2 (40)	12 (39)	2 (25)	2 (20)	6 (60)
Ever surgery	0 (0)	2 (25)	1 (20)	12 (39)	2 (25)	7 (70)	5 (50)
Colectomy	0 (0)	1 (13)	1 (20)	7 (23)	0 (0)	0 (0)	0 (0)
Medication							
Ever TNF-inhibitor	0 (0)	2 (25)	1 (20)	4 (13)	0 (0)	1 (10)	1 (10)
Ever glucocorticoids	0 (0)	3 (38)	4 (80)	17 (55)	2 (25)	6 (60)	6 (60)
Ever other immunosupressor[3]	0 (0)	4 (50)	2 (40)	6 (19)	0 (0)	2 (20)	5 (50)
Ever 5-ASA	1 (2)	5 (63)	4 (80)	22 (71)	1 (13)	5 (50)	6 (60)
Smoking							
Never smoker	28 (54)	6 (75)	2 (40)	21 (68)	5 (63)	3 (30)	2 (20)
Current smoker	10 (19)	1 (13)	1 (20)	2 (6)	2 (25)	3 (30)	7 (70)
Former smoker	13 (25)	1 (13)	2 (40)	7 (23)	1 (13)	4 (40)	1 (10)

[1]Fistula, adherences, strictures, toxic megacolon, abscess, perforation, colorectal cancer; [2]Hepatitis, primary sclerosing cholangitis, autoimmune pancreatitis, uveitis, erythema nodosum, pyoderma gangrenosum, arthritis, aphthous ulcers; [3]Methotrexate, azathioprine, and cyclosporine;. *n* denotes the number of participants with the phenotype described in status. IBD: Inflammatory bowel diseases; CD: Crohn's disease; UC: Ulcerative colitis.

The 8 cases were afflicted by the following diagnoses: lymphocytic colitis, irritable bowel syndrome, *Clostridium Difficile* infection, ischemic bowel changes and abscesses without pathologic CD features and grouped as GI symptoms not IBD. At least one twin suffered from IBD in all remaining 54 pairs according to patient records before verification of diagnostic criteria. Forty-four were discordant for IBD, of whom 24 out of 31 UC pairs, four out of five IBDU pairs, and six out of eight CD pairs fulfilled the Copenhagen diagnostic criteria. Of the 10 concordant pairs, there were eight CD/CD pairs, one UC/UC pair, and one UC/IBDU pair, where all but one CD index twin fulfilled the Copenhagen criteria. Both verified and suspected cases were included in the cohort, to reflect clinical practice.

Age at diagnosis

The mean age at diagnosis was lower in the CD concordant than CD discordant pairs (24.75 years *vs* 31.75 years). The timespan between the diagnosis of an index twin and the IBD co-twin was 6 years on average, ranging from 94 d to 14 years. The mean disease duration at sampling was 15 years on average, ranging from 295 d to 37 years.

Clinical characteristics, complications, medication and smoking

Table 1 shows clinical characteristics of the discordant twin pairs. Nine extra intestinal manifestations were present among co-twins, most often arthropathy. Table 2 shows complications, medication and smoking. Though numbers are small, 25% of CD index twins and 63% of CD co-twins received surgery after their IBD diagnosis. Conversely, 50% received azathioprine among the CD index twins *vs* 13% among the CD co-twins.

Assessment of inflammatory activity in concordant and discordant twin pairs

Figure 2A shows inflammatory activity in discordant co-twin pairs as measured by calprotectin, at the time of sample collection. There was evidence of gut inflammation in the apparently non-affected co-twin, with faecal calprotectin > 100 μg/g in two individuals and > 50 μg/g in a further five (Figure 2A). In two of the index twins whose IBD diagnosis could not be verified, faecal calprotectin was > 100 μg/g. Values of patient reported disease scores were also slightly increased though slightly less pronounced (Figure 2B and C).

DISCUSSION

We have established a nationwide cohort of 62 affected or suspected IBD monozygotic twin pairs, which allow assessment of a range of disease- and treatment-associated and phenotypical traits amongst both discordant and concordant MZ IBD twins. Validation of the CD, UC, and IBDU diagnoses resulted in 8 pairs where the diagnosis was unlikely, and 11 pairs where the diagnosis was likely, but the clinical information was too sparse to validate this. Therefore, 43 twin pairs fulfilled the Copenhagen diagnostic criteria. The cohort included 10 concordant pairs, and several of these IBD concordant pairs were discordant for either treatment or surgery. The 44 IBD discordant pairs comprised 31 UC pairs, five IBDU pairs, and eight CD pairs. Inflammatory activity was above the normal range in 7 of the co-twins not affected by IBD, with calprotectin above 100 μg/g in two co-twin pairs and above 50 μg/g in a further five pairs.

The strength of the presented twin cohort lies in the wide range of data collected, from questionnaire

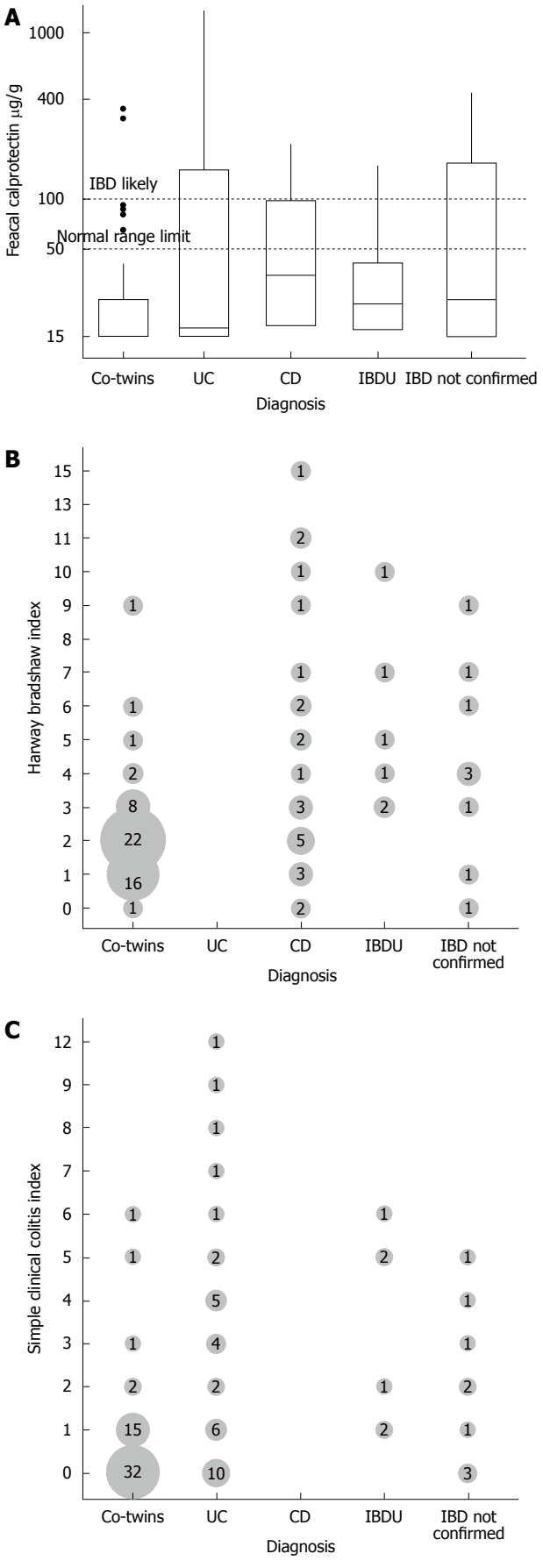

Figure 2 Figure shows fecal calprotectin measures stratified (A), harwey bradshaw index stratified by phenotype (B) and simple clinical colitis index stratified (C) by phenotype. IBD: Inflammatory bowel diseases; CD: Crohn's disease; UC: Ulcerative colitis.

data, patient file and public register data, to multiple biological samples. Our mobile laboratory enabled uniform collection of biological material with few deviations from existing guidelines regarding sample collection, storage, and handling. The average time from sampling to storage at -80 ℃ was 1 h or less for all samples. Our uniform sample collection method using a mobile laboratory reduced aberrant data handling normally affecting nationwide multicentre studies, and allowed for a single-site analysis of disease activity data. A drawback of this approach is that more advanced laboratory handling, like cell separation and preserving viable cells, was not performed. Instead, the study used CPT tubes, a commercial cell preservative, and gradual freezing of cells using a "mister frosty", which has previously been shown to preserve viable cells and cell integrity[34].

While the collection of biological material in this study is more uniform than previous twin studies[14,35-40], we were unable to perform invasive manoeuvres such as endoscopy with our mobile setup. Though we expect to achieve access to some biopsy material taken from routine endoscopies, a large proportion of the healthy twins had not recently undergone endoscopy, thus limiting opportunities for comparison. Our assessment of clinical characteristics and IBD medication use aggregated data from patient files and questionnaire data. Consequently, treatment not documented by hospital-based physicians or recalled by patients may remain unaccounted for, but this potential bias should be similar between concordant and discordant pairs.

Our inclusion rate was lower than expected at 62 pairs out of the contacted 159, perhaps due to the extent of collected samples, and the need for including both twins. Indeed, some selection bias favouring the inclusion of concordant pairs over discordant pairs could not be ruled out, based on the proportion of concordant pairs invited to the proportion of concordant pairs accepting to participate.

The IBD twins were identified using nationwide registers, reducing bias often bestowed upon twin studies relying on advertising for recruitment. Given sufficient power, concordant pairs may play a crucial role in discerning the effects of disease-associated traits, such as medical therapy, from the effects of IBD, *e.g.,* on the methylome or metagenome. In addition, though we did not have the power to test this formally, the mean age at diagnosis seemed lower in the CD concordant pairs at 25 years *vs* 32 years among CD discordant pairs. Results from previous twin studies are conflicting on this point[41-43]. If indeed such a difference exists, one possible explanation might be that concordant pairs carry a larger genetic liability to disease, with a lower threshold for disease throughout life, increasing the risk of both twins contracting IBD, resulting in twin concordance. A previous Swedish twin study found the total allele frequency of *Nod/Card* mutations to be 4.4 times higher among concordant twin pairs compared to discordant twin pairs contributing to, but not explaining concordance[41].

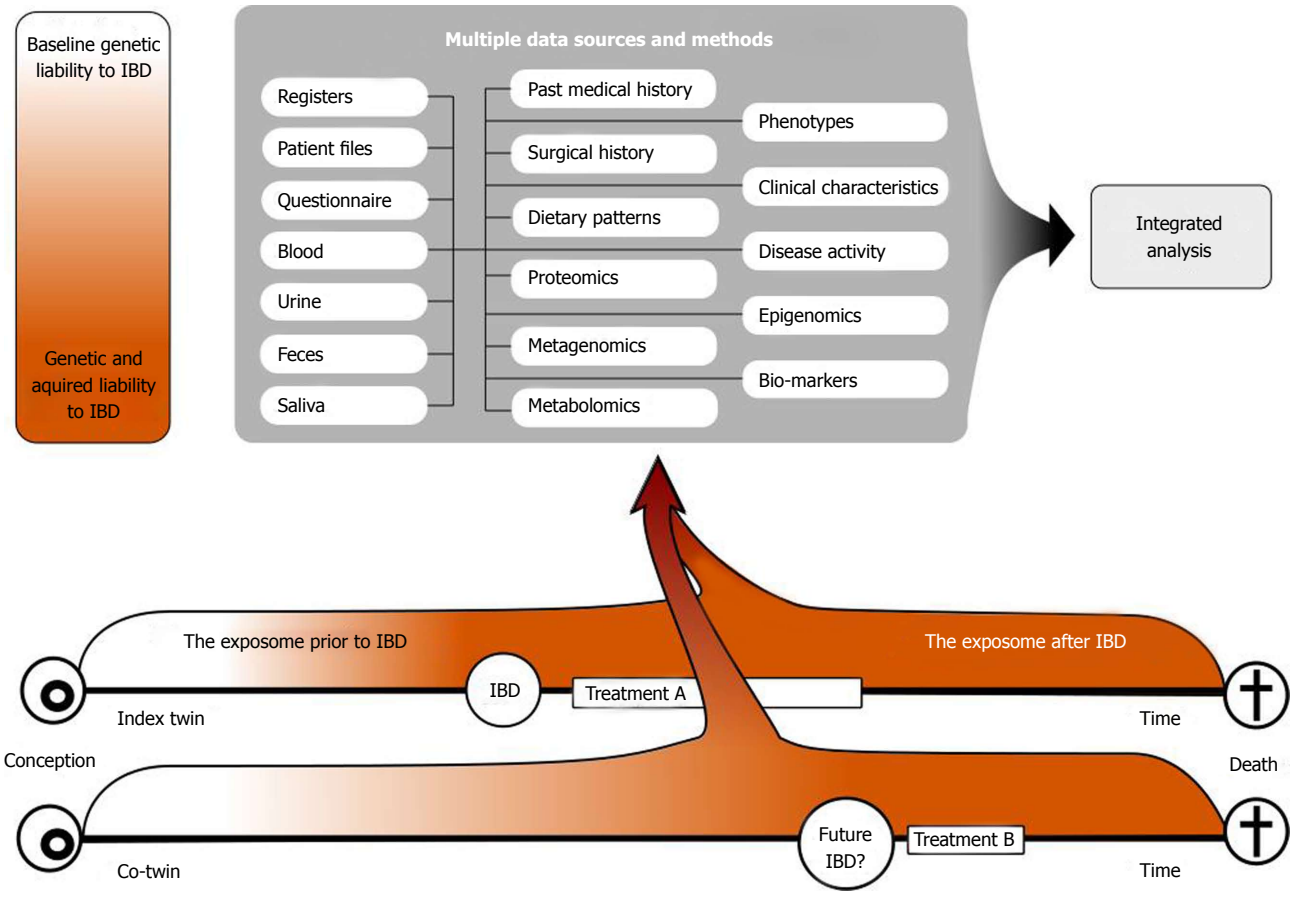

Figure 3 Figure shows how the collected twin data may be used in different downstream analyses. The figure illustrates the initial genetic concordance in liability, the progressive discordance for disease liability due to heterogeneous exposures, and the possible future concordance for IBD, but not necessarily for treatment. IBD: Inflammatory bowel diseases.

Phenotypical characteristics differentiate CD and UC from each other and from other conditions such as IBS, microscopic colitis and infections. A combination of clinical evaluation, endoscopic, histological, radiological, and/or bio-chemical investigations provides the diagnostic foundation for CD and UC[44,45]. The correct classification of twin discordance is paramount. Of note, classification is not only dependent on the correct diagnosis but also on time interval following the diagnosis of the index twin, with the risk of contracting IBD declining with time for the co-twin. Although the maximum time-span between concordant pairs in this cohort was below the mean disease duration of 15 years in the present cohort, final verification of twin discordance can only be assessed at the end of the lifespan of both twins. Methods do however exist that take time to event into account[46]. While future disease may not be a problem in a discordant twin study design, this is only true if the exposures causing this disease are not already present. Quiescent and subclinical disease may complicate the distinction between cases and their co-twin controls. Indeed, a newly published study indicates the presence of latent or emerging disease in family members of affected IBD cases[16]. In addition, family members of affected IBD cases have increased calprotectin levels as compared to the background population[17]. Though a normal calprotectin level does not exclude IBD, due to the dynamic nature of this condition, and a low level in well treated IBD patients, calprotectin levels correlate with intestinal inflammation[18-20], and could thus reflect an increased liability to IBD in familial members of IBD-affected cases[47,48]. One[48] twin study published in the past 10 years has reported on increased levels of intestinal inflammatory activity biomarkers such as calprotectin among the unaffected co-twins, while the majority of previous twin studies have not[14,35-40,49]. We found two co-twins with no history of IBD with calprotectin values exceeding 100 μg/g, and a further 5 with values above the normal range. This may be important, as increased calprotectin may reflect that many of the exposures leading to disease may already be present in a co-twin, if subclinical disease is not already present. As a result, inter-individual differences with impact on disease pathogenesis within pairs may be harder to assess, suggesting that calprotectin levels should be considered in analysis.

Disease discordant IBD twins remain rare and precious to research[14,35-40,49]. Though providing a powerful model for research, this will often mean insufficient power for planned analyses. Hence, collaboration between twin resources is crucial. Collaboration with

the Nixon Twin and Multiplex (TAM) United Kingdom IBD cohort, analysing epigenetic data within similar biological material, has already been established[50]. Thus, both the Danish and the British IBD twin cohorts will include a range of clinical, epidemiological and biological data enabling researchers to study a cross section of the IBD exposome (Figure 3).

The present cohort demonstrates the importance of assessing inflammatory biomarkers reflecting subclinical inflammatory activity among otherwise healthy co-twins in discordant twin studies. The present cohort will be part of international collaborations, thereby increasing the power to detect disease-associated factors, and allow sufficient concordant twins to be included in studies to adjust for treatment effects. Hypotheses that may be tested include whether epigenetic differences controlling IBD loci previously identified in GWAS studies exists within the twin pairs. Other approaches may include rodent models where rodent responses to biological material from discordant pairs may differ. Consequently, analysis of a range of data from cohorts of monozygotic IBD pairs using bioinformatic methods such as metagenomics, metabolomics, proteomic and epigenetics could provide new insight into the role of the exposome in IBD pathogenesis.

COMMENTS

Background

Although studies of the genome have found 200 loci associated with inflammatory bowel diseases (IBD), the variation in the IBD phenotype explained by these finding is still below 25%-30%, suggesting a role of environmental factors in IBD pathogenesis. Several studies indicate environmental impact on IBD pathogenesis, including exposure to pathogens, disease-associated dysbiosis, metabolic disequilibrium, or epigenetic modifications. Comprehensive studies of the exposome using IBD discordant MZ twin co-twin study designs could prove a powerful tool to assess the combined effects of environmental and endogenous factors, and identify targets for treatment and prevention.

Research frontiers

Historically twin studies have been used to calculate the heritability of complex traits and diseases. The co-twin control design constitutes an excellent model to investigate environmental factors associated with disease due to the genetic match between monozygotic twins. To date only a few studies have applied this method using bioinformatics methods in IBD. Most prominent is the work of Jonas Halvfosen and his group in Orebro Sweden that identified differential microbial stool patterns between IBD discordant twin pairs, underlining the potential of this methodology.

Innovations and breakthroughs

Co-twin control designs may result in complexity reduction, thus increasing power to identify microbial or epigenetic patterns associated with IBD and the interplay between these complex traits. Such studies necessitate cohorts as the one described in this study designed for downstream bioinformatics studies, and special emphasis was on pre-analytical sample handling.

Applications

The present cohort demonstrates the importance of assessing inflammatory biomarkers reflecting subclinical inflammatory activity among otherwise healthy co-twins in discordant twin studies. Using co-twin study designs to investigate environmental determinants of disease holds promise for future discovery. However, as MZ IBD discordant twins are rare this often means insufficient statistical power. Hence, collaboration between twin resources is crucial. Through international collaborations analysis of a range of data from cohorts of monozygotic IBD pairs using bioinformatic methods such as metagenomics, metabolomics, proteomics and epigenetics could provide new insight into the role of the exposome in IBD pathogenesis.

Terminology

Concordant twin pairs: twin pairs where both twins are affected by disease or trait. Discordant twin pairs: twin pairs where only one twin is affected by disease or trait. According to Wild (2005), the exposome encompasses all human environmental exposures from conception onwards.

Peer-review

As the authors realize, the real strength of this cohort is in the future translational studies, primarily as it relates to epigenetics. While they very briefly and superficially discuss these plans in the last paragraph of the conclusion, expanding on future plans for hypothesis-driven translational research would further strengthen the manuscript. Otherwise, this is a nice introduction to a novel cohort that hopes to generate fascinating future work.

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ORIGINAL ARTICLE

Case Control Study

Serum *Helicobacter pylori* KatA and AhpC antibodies as novel biomarkers for gastric cancer

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Abstract

AIM: To investigate catalase (KatA) and alkyl hydroperoxide reductase (AhpC) antibodies of *Helicobacter pylori* as biomarkers for gastric cancer (GC).

METHODS: This study included 232 cases and 264 controls. Recombinant KatA and AhpC proteins were constructed and the levels of antibodies were tested by indirect enzyme-linked immunosorbent assay (ELISA). Logistic regression was applied to analyze the relationships between KatA, AhpC and GC. The χ^2 trend test was used to evaluate the dose-response relationships between serum KatA and AhpC antibody levels and GC. Receiver operating characteristic (ROC) curve was used to evaluate the screening accuracy of KatA and AhpC as biomarkers. Combined analysis was used to observe screening accuracy of predictors for GC.

RESULTS: In all subjects, the association between KatA and AhpC and GC risk was significant ($P < 0.001$) with odds ratio (OR) = 12.84 (95%CI: 7.79-21.15)

and OR = 2.4 (95%CI: 1.55-3.73), respectively. KatA and AhpC antibody levels were strongly related to GC risk with a dose-dependent effect (*P* for trend < 0.001). The area under the ROC (AUC) for KatA was 0.806, providing a sensitivity of 66.81% and specificity of 86.36%; and the AUC for AhpC was 0.615, with a sensitivity of 75.65% and specificity of 45.49%. The AUC was 0.906 for KatA and flagella protein A (FlaA) combined analysis.

CONCLUSION: Serum KatA and AhpC antibodies are associated with GC risk and KatA may serve as a biomarker for GC. KatA/FlaA combined analysis improved screening accuracy.

Key words: *Helicobacter pylori*; Catalase; Serum antibody; Alkyl hydroperoxide reductase; Gastric cancer; Case-control study

Core tip: Effective screening methods for gastric cancer (GC) have remained limited to date. The aim of this study was to explore whether serum catalase and alkyl hydroperoxide reductase antibodies of *Helicobacter pylori* could serve as novel and reliable biomarkers for GC monitoring.

Zhang B, Li HL, Fan Q, Guo F, Ren XY, Zhou HB, Zhu JW, Zhao YS, Tian WJ. Serum *Helicobacter pylori* KatA and AhpC antibodies as novel biomarkers for gastric cancer. *World J Gastroenterol* 2016; 22(21): 5060-5067 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i21/5060.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i21.5060

INTRODUCTION

Gastric cancer (GC) is the fifth most common malignancy and the third leading cause of cancer-related death worldwide[1]. Although the overall incidence rate of GC continues to fall, there were still almost 1 million new cases of GC in 2012[2]. *Helicobacter pylori (H. pylori)* are micro-aerophilic gram-negative bacteria that cause inflammatory reactions by selectively colonizing the gastric mucosa. The International Agency for Research on Cancer has classified *H. pylori* as a category I carcinogen since 1994[3]. Epidemiological data also support that *H. pylori* infection is strongly associated with GC[4-6], increasing risk by up to six-fold[7]. In contrast, increasing data shows that *H. pylori* eradication significantly decreases the development of GC[8,9], particularly in high-risk populations with no precancerous lesions[10]. Eradication of *H. pylori* seems a reasonable approach for preventing GC. However, nearly 50% of the population worldwide is infected with *H. pylori*[11]. Mass eradication therapy in the general population may bring about development of antibiotic-resistant strains of *H. pylori* as well as over-consumption of medical resources. Therefore, there is an urgent and need to identify a reliable screening biomarker for GC.

It is reported that only a small fraction of patients infected with *H. pylori* have severe clinical outcomes, such as gastric ulcer (10%), atrophic gastritis (5%), and gastric malignancy (2%)[3]. Research indicates that these different outcomes may be associated with the virulence factors of *H. pylori*[12-14]. Catalase (KatA) and alkyl hydroperoxide reductase (AhpC) virulence factors play a crucial role in protecting *H. pylori* from oxidative stress and maintaining a stable environment for the growth of bacteria[15,16]. Huang *et al*[17] confirmed that KatA and AhpC were over expressed under the condition of oxidation stress (H_2O_2) in *H. pylori* strains isolated from patients with GC, gastritis, or duodenal ulcer. We previously reported that serum flagella protein A (FlaA) antibody of *H. pylori* may serve as noninvasive biomarker for early detection of GC[18]. In this study, combined analysis was applied to explore the screening value of KatA, AhpC, and FlaA for GC. This study aims to assess the correlations between KatA and AhpC and GC and explore whether they could serve as novel and reliable biomarkers for GC.

MATERIALS AND METHODS

Study subjects

This was a hospital-based case-control study, which was approved by the Committee of Human Research of Harbin Medical University, Harbin, China. Two hundred and thirty-two cases of GC were primarily diagnosed by pathology at the Third Affiliated Hospital of Harbin Medical University between April and July 2010. The controls comprised 182 healthy people chosen from the Harbin Xiangfang Center for Disease Control and Prevention and 82 cancer-free people recruited from the neurology department at the Fourth Affiliated Hospital of Harbin Medical University between March and July 2011. All participants gave signed informed consent, and we completed a face-to-face questionnaire that included age, sex, smoking status, and alcohol consumption. Venous blood samples of 5 mL were collected from all participants, centrifuged at 3000 r/min, and stored at -80 ℃.

Cloning and expression of recombination protein

A clinical strain of *H. pylori* provisionally named H015a was isolated from a GC patient at the Second Affiliated Hospital of Harbin Medical University. Genomic DNA of H015a was extracted as a template using a DNA extraction kit (QIAGEN, Valencia, CA, United States). The *katA* and *ahpC* gene coding sequences were obtained from Genbank. Amplification of *katA* and *ahpC* gene fragments was implemented by polymerase chain reaction (PCR). The PCR primers were designed using Primer Premier 5.0 software. For *katA*, the primer sequences were 5'-CCGGAATTCATGGTTAATAAAGATGTGAACA-3'

(forward) and 5′-CCGCTCGAGTTACTTTTTCTTTTT-TGTGTGG-3′ (reverse) that generated a 1518 bp fragment. For *ahpC*, the primer sequences were 5′-CCGGAATTCATGTTAGTTACAAAACTTGCCC-3′ (forward) and 5′-CCGCTCGAGTTAAAGCTTAATG-GAATTTTC-3′ (reverse) that generated a 597 bp fragment. *Eco*RI and *Xho*I restriction endonuclease sites were incorporated into the forward and reverse primer sequences of these two genes, respectively. Amplification was implemented under the following conditions: 30 cycles of 94 ℃ for 30 s, 55 ℃ for 30 s, and 72 ℃ for 90 s followed by a final extension at 72 ℃ for 7 min. Subsequently, two PCR products were cloned into the cloning vector pMD18-T and transformed into *Escherichia coli* (*E. coli*) strain DH5α. The positive clones were screened and cloned into the prokaryotic expression vector pET-32a. The recombinant plasmids *katA*-pET-32a and *ahpC*-pET-32a were introduced into *E. coli* BL21 (DE3) cells for expression of recombinant proteins, respectively. The target sequences of *katA* and *ahpC* gene were assayed by the dideoxy chain termination method (Biotechnology firm, BGI, Beijing, China). The recombinant *katA*-pET-32a-BL21 and *ahpC*-pET-32a-BL21 strains were cultured in lysogeny broth (LB) with 100 μg/mL ampicillin, and induced at 30 ℃ by isopropylthio-β-d-galactoside with a final concentration of 1 mmol/L and 0.5 mmol/L, respectively. *E. coli* cells were harvested after 4 h and disrupted ultrasonically. The suspension and precipitate were collected and protein expression was analyzed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

H. pylori serological tests

A serological test for *H. pylori* immunoglobulin (Ig)G antibodies has already been completed and described by our group[17].

Purification and renaturation of target recombinant proteins

The recombinant proteins were purified by Ni-NTA His Bind resin (Novagen, Darmstadt, Germany). We used stepwise dialysis to obtain the fusion protein by removing the denaturant (urea) in the purified protein. The dialysis tube was boiled 10 min in buffer (2% $NaHCO_3$ and 1mmol/L EDTA pH 8.0) and EDTA solution (1 mmol/L) sequentially. After cooling, the purified protein was put into the dialysis tube, and both ends were clamped with the dialysis clips. The protein was dialyzed in urea solution (pH 8.3) with a slowly decreasing concentration: 6 mol/L, 4 mol/L, 2 mol/L, 1 mol/L and 0 mol/L. Each dialysis lasted 24 h. Finally, the sample was removed from the dialysis tube and stored at -80 ℃ until analysis.

Detection of antibodies against recombinant proteins with enzyme-linked immunosorbent assay

An indirect enzyme-linked immunosorbent assay (ELISA) was applied to detect the serum antibodies against *H. pylori* recombinant KatA and AhpC proteins. Recombinant KatA and AhpC proteins were diluted to 2 μg/mL and 0.25 μg/mL, respectively. Proteins at 100 μL/well were incubated in a 96-well micro-plate (Costar, Washington, DC, United States) at 4 ℃ overnight and washed three times with phosphate buffer saline, Tween-20 (PBST), followed by blocking with 10% goat serum (AR0009; Boster, Beijing, China) and incubation for 2 h at 37 ℃. Serum sample from cases and controls diluted 3200-fold with 10% bovine serum albumin (BSA) was added to the plate at 100 μL/well and incubated for 1 h at 37 ℃. Each serum sample was tested in three parallel wells. The plate was again washed three times with PBST. Peroxidase-conjugated goat anti-human IgG (H+L) (ZSGB-Bio, Beijing, China) was diluted 1:5000 with buffer, and 100 μL was added to each well and incubated 30 min at 37 ℃. Tetramethylbenzidene (TMB) substrate buffer was added to the plate at 100 μL/well and incubated in the dark for 15 min at 37 ℃, Fifty microliters stop solution was added per well to terminate the reaction. Finally, the plate was read at 450 nm absorbance using a micro-plate reader (Biotech Synergy 2, Winooski, Vermont, United States). The determination of serostatus of antibody was based on optical density (OD) value. The optimal cutoff point of OD values was used to classify samples as seropositive or seronegative.

Statistical analysis

All statistical analyses were conducted using SPSS 22.0 version software (Armonk, NY, United States). Unconditional logistic regression analysis was performed to estimate odds ratio (OR) and 95% confidence interval (CI) for the relationship between GC and antibodies. The χ^2 trend test was used to assess dose-response relationships between serum KatA and AhpC antibody levels and GC. In addition, a receiver operating characteristic (ROC) curve was plotted to identify the cutoff point of serum KatA and AhpC antibody results. Sensitivity, specificity, and area under the ROC curve (AUC) with 95%CI were calculated to evaluate the screening value of serum KatA and AhpC antibody levels for GC. Moreover, the optimal cutoff value was determined by the maximum Youden index (Youden index = sensitivity + specificity - 1). Combined analysis was used to observe screening accuracy of predictors for GC. For all tests, $P < 0.05$ was considered statistically significant.

RESULTS

Characteristics of study subjects

The characteristics of the study subjects were described in our previous study[18].

Cloning and expression of the recombinant proteins

Nucleotide homology of the cloned *katA* gene compared to *H. pylori 26695* was 95.52% The homology of

Table 1 Association between gastric cancer and seropositivity of catalase and alkyl hydroperoxide reductase antibodies in study subjects *n* (%)

Virulence factors serostatus	All subjects				*H. pylori* positive subjects				*H. pylori* negative subjects			
	Case	Control	OR (95%CI)	*P* value[1]	Case	Control	OR (95%CI)	*P* value[1]	Case	Control	OR (95%CI)	*P* value[1]
KatA												
Negative	78 (33.62)	228 (86.36)	1.0 (Reference)	< 0.001	47 (35.61)	104 (88.14)	1.0 (Reference)	< 0.001	26 (29.21)	109 (83.85)	1.0 (Reference)	< 0.001
Positive	154 (66.38)	36 (13.64)	12.84 (7.80-21.15)		85 (64.39)	14 (11.86)	14.59 (6.84-31.13)		63 (70.79)	21 (16.15)	12.15 (5.79-25.51)	
AhpC												
Negative	56 (24.14)	121 (45.83)	1.0 (Reference)	< 0.001	33 (25.00)	57 (48.31)	1.0 (Reference)	< 0.001	54 (54.00)	103 (70.55)	1.0 (Reference)	< 0.001
Positive	176 (75.86)	143 (54.17)	2.40 (1.55-3.73)		99 (75.00)	61 (51.69)	2.30 (1.25-4.23)		46 (46.00)	43 (29.45)	2.04 (1.10-3.78)	
Combination of KatA and AhpC												
Negative	78 (33.62)	226 (85.61)	1.0 (Reference)	< 0.001	49 (37.12)	104 (88.14)	1.0 (Reference)	< 0.001	33 (33.00)	127 (86.99)	1.0 (Reference)	< 0.001
Positive	154 (66.38)	38 (14.39)	11.64 (7.12-19.01)		83 (62.88)	14 (11.86)	13.40 (6.29-28.53)		67 (67.00)	19 (13.01)	13.91 (6.74-28.74)	

[1]The *P* value was obtained from logistic regression analysis adjusted for age, sex, family history of gastric cancer, smoking, and alcohol consumption. KatA: Catalase; AhpC: Alkyl hydroperoxide reductase.

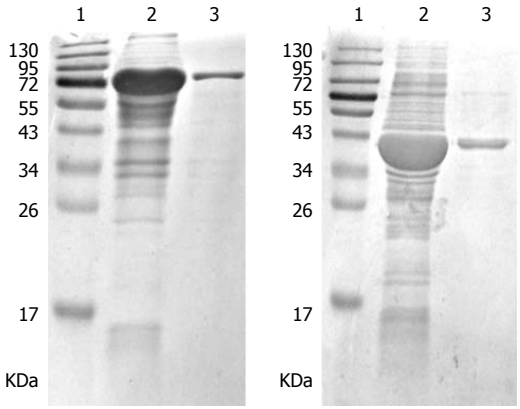

Figure 1 SDS-PAGE analysis of purified recombinant proteins. A: KatA; B: AhpC. 1: Marker; 2: Unpurified protein; 3: Purified protein. KatA: Catalase; AhpC: Alkyl hydroperoxide reductase.

ahpC nucleotide was 96.48% compared with *H. pylori J99.*

A prokaryotic expression system was constructed. After induction by isopropyl beta D thiogalactoside (IPTG), proteins with the expected size were clearly present as inclusion bodies in the ultrasonic precipitation by SDS-PAGE. Finally, the purified fusion proteins were obtained (Figure 1).

Association between serum positivity of antibodies and GC

As shown in Table 1, an association between KatA and GC risk was observed, with OR = 12.84 (95%CI: 7.79-21.15), 14.59 (6.84-31.13), and 12.15 (5.79-25.51) in all, *H. pylori*-positive and *H. pylori*-negative subjects, respectively ($P < 0.001$). Dose-dependent effects showed that KatA antibody levels were strongly related to GC risk in the three populations mentioned above (P for trend < 0.001) (Table 2). Similarly, a significant association between GC risk and serum positivity of AhpC was observed with OR = 2.40 (95%CI: 1.55-3.73) in all subjects, 2.30 (1.25-4.23) in *H. pylori*-positive subjects, and 2.04 (1.10-3.78) in *H. pylori*-negative subjects ($P < 0.001$) (Table 1). Correspondingly, AhpC antibody level was significantly related to GC risk in a dose-dependent manner (P for trend < 0.001) (Table 2). Moreover, an evident association between GC risk and serum positivity of combination of KatA and AhpC was present, with OR = 11.64 (95%CI: 7.12-19.01), 13.39 (6.29-28.53), and 13.91 (6.74-28.74) in all, *H. pylori*-positive and *H. pylori*-negative subjects, respectively ($P < 0.001$) (Table 1).

Screening utility of serum antibody for GC

An ROC curve was plotted to explore the screening value of KatA and AhpC for GC. The AUC for KatA was 0.806 (95%CI: 0.768-0.845), 0.805 (0.751-0.853), and 0.801 (0.741-0.861) in all, *H. pylori*-positive and *H. pylori*-negative subjects, respectively (Figure 2). The AUC for AhpC was 0.615 (95%CI: 0.566-0.665) in all subjects, 0.629 (0.560-0.699) in *H. pylori*-positive subjects, and 0.605 (0.530-0.680) in *H. pylori*-negative subjects (Figure 3). As shown in Table 3, the optimal cutoff value of KatA and AhpC for GC was 0.3583 and 0.3647 in all subjects, providing a sensitivity of 66.81% and 75.65% and a specificity of 86.36% and 45.49%, respectively. AUC for the combination of KatA and FlaA was 0.906 (95%CI: 0.879-0.932), and the optimal cutoff value was 0.4305 with a sensitivity of 78.88% and a specificity of 89.02% (Figure 4A). For combination of KatA, FlaA, and AhpC, the AUC was 0.910

Table 2 Dose-dependent association between gastric cancer risk and serum catalase and alkyl hydroperoxide antibodies levels in study subjects *n* (%)

All subjects					*H. pylori* positive subjects					*H. pylori* negative subjects				
Antibody level (OD)[1]	Case	Control	OR (95%CI)[2]	*P* value for trend	Antibody level (OD)	Case	Control	OR (95%CI)[2]	*P* value for trend	Antibody level (OD)	Case	Control	OR (95%CI)[2]	*P* value for trend
KatA														
≤ 0.4187	167 (71.98)	66 (25.0)	1.0 (Reference)	< 0.001	≤ 0.4152	92 (69.70)	29 (24.58)	1.0 (Reference)	< 0.001	≤ 0.4167	66 (74.16)	32 (24.58)	1.0 (Reference)	< 0.001
0.4187-0.5313	36 (15.52)	66 (25.0)	4.25 (2.49-7.27)		0.4152-0.5133	23 (17.42)	30 (25.42)	3.79 (1.78-8.06)		0.4167-0.5568	9 (10.11)	33 (25.42)	6.67 (2.70-16.51)	
0.5313-0.6799	18 (7.76)	66 (25.0)	9.95 (5.05-19.62)		0.5133-0.6692	9 (6.82)	30 (25.42)	9.69 (3.81-24.70)		0.5568-0.6824	11 (21.36)	33 (25.42)	7.00 (2.68-18.30)	
> 0.6799	11 (4.74)	66 (25.0)	15.85 (6.97-36.06)		> 0.6692	8 (6.06)	29 (24.58)	16.55 (5.51-49.76)		> 0.6824	3 (3.39)	32 (24.58)	19.89 (4.32-91.70)	
AhpC														
≤ 0.2168	88 (37.93)	66 (25.00)	1.0 (Reference)	< 0.001	≤ 0.2182	51 (38.64)	29 (24.58)	1.0 (Reference)	< 0.001	≤ 0.2110	31 (34.83)	32 (24.58)	1.0 (Reference)	< 0.001
0.2168-0.3265	69 (29.74)	66 (25.00)	1.26 (0.76-2.11)		0.2182-0.3433	41 (31.06)	30 (25.42)	1.10 (0.54-2.25)		0.2110-0.3310	29 (32.58)	33 (25.42)	1.44 (0.69-3.00)	
0.3265-0.4888	49 (21.12)	66 (25.00)	1.41 (0.82-2.43)		0.3433-0.4908	28 (21.21)	30 (25.42)	1.54 (0.70-3.38)		0.3310-0.4948	16 (17.98)	33 (25.42)	1.83 (0.80-4.23)	
> 0.4888	26 (11.21)	66 (25.00)	3.54 (1.84-6.82)		> 0.4908	12 (9.09)	29 (24.58)	3.40 (1.32-8.73)		> 0.4948	13 (14.61)	32 (24.58)	3.33 (1.31-8.46)	

[1]Serum positivity for the antibodies to KatA and AhpC was categorized by quartiles of antibody levels in controls; [2]Adjusted for age, sex, family history of gastric cancer, smoking, and alcohol consumption. *H. pylori*: *Helicobacter pylori*; KatA: Catalase; AhpC: Alkyl hydroperoxide reductase.

(95%CI: 0.885-0.935), offering a sensitivity of 80.17% and a specificity of 88.64%, while the optimal cutoff value was 0.4354 (Figure 4B).

DISCUSSION

Gastric carcinogenesis is a multifactorial process, and *H. pylori* infection plays an important role in the initial stage[19]. Patients with malignant tumors are often diagnosed at an advanced stage, and 5-year survival rate is < 10%[20]. Therefore, early detection is a crucial factor for GC prevention. However, it is difficult to diagnose GC any earlier because the symptoms of gastric pre-cancerous and malignant diseases are non-specific and vague. At present, endoscopy is the gold standard for screening GC and is commonly used in the clinic. A large case-control study from Japan indicated that GC mortality was reduced 30% by endoscopic screening compared with no screening[21]. In spite of this finding, limitations of endoscopy, such as the existence of over diagnosis and unwillingness of asymptomatic patients because of pain as well as cost make endoscopy unsuitable for population-based screening. Serological testing is widely available and is a low-cost noninvasive diagnostic method. In the present study, we explored whether serum *H. pylori* antibody could serve as a biomarker for GC monitoring.

KatA is a ubiquitous enzyme that protects *H. pylori* cells from extracellular H_2O_2 attack[22,23] and plays an important role in colonization of gastric mucosa[15]. AhpC is the most abundant and essential antioxidant protein of *H. pylori*[16], and it protects bacteria from lipid peroxidation and DNA damage[24,25]. We used a commercial ELISA method to detect *H. pylori* infection status. However, this method may fail to detect prior *H. pylori* infection in GC patients, and patients positive for anti-CagA (cytotoxin-associated gene A) antibody may have negative results for *H. pylori* serological testing[26,27]. In order to eliminate these possible influences on our results, *H. pylori*-negative and overall subjects were also analyzed to observe the associations between GC and the KatA and AhpC antibodies. The results indicated that we should be more vigilant regarding antibody titer and seropositivity. Meanwhile, we found that the median of KatA and AhpC antibody levels were lower in cases group than in the controls (data not shown). This finding implied that the high antibody titer of *H. pylori* KatA and AhpC may protect against the occurrence of GC.

A Latin American study showed that seropositivity of KatA in a population within a high risk of GC area was higher than that in a low-risk population[28]. Our results confirmed that KatA was associated with GC, and seropositivity of KatA antibody showed a 14.59-fold increased risk of GC. Yan *et al*[29] found that AhpC antibody of *H. pylori* may be related to the development of gastric diseases using the gerbil model to simulate human *H. pylori* infection. In addition, Huang *et al*[30] indicated that AhpC was expressed in greater amounts in GC than gastritis strains. In our study, there was a significant association between AhpC antibody and GC, based on epidemiology data. Further analysis found that KatA and AhpC antibody levels were strongly related to GC risk in a dose-dependent manner. In order to explore whether KatA and

Table 3 Sensitivity and specificity of different catalase and alkyl hydroperoxide reductase critical values

Percentile[1]	All subjects			*H. pylori* positive subjects			*H. pylori* negative subjects		
	Critical value (OD)[2]	Sensitivity	Specificity	Critical value (OD)[2]	Sensitivity	Specificity	Critical value (OD)[2]	Sensitivity	Specificity
KatA									
Optimal cutoff point[2]	0.3583	66.81%	86.36%	0.3557	64.39%	88.14%	0.3730	70.79%	83.85%
25%	0.2800	46.55%	93.18%	0.4152	69.70%	74.58%	0.2773	50.56%	90.00%
50%	0.4305	75.00%	71.59%	0.5133	87.12%	49.15%	0.4447	78.65%	69.23%
75%	0.5958	90.95%	36.36%	0.6692	93.94%	24.58%	0.6107	92.13%	36.15%
90%	0.7418	97.84%	16.29%	0.9042	100.00%	8.47%	0.7873	97.75%	14.62%
AhpC									
Optimal cutoff point[2]	0.3647	75.65%	45.49%	0.3613	75.76%	48.31%	0.2330	43.82%	70.77%
25%	0.1953	30.43%	78.95%	0.1953	30.30%	80.51%	0.1917	30.34%	77.69%
50%	0.2830	59.57%	57.14%	0.2865	59.85%	60.17%	0.2913	62.92%	57.69%
75%	0.4267	84.35%	32.71%	0.4325	83.33%	33.05%	0.4313	85.39%	23.85%
90%	0.5747	95.65%	14.29%	0.5302	93.94%	13.56%	0.6410	96.63%	13.85%

[1]Percentiles of serum KatA and AhpC antibody levels in controls; [2]Optimal cutoff point in the different parameters was identified according to the maximum Youden's index (sensitivity + specificity - 1). *H. pylori*: *Helicobacter pylori*; KatA: Catalase; AhpC: Alkyl hydroperoxide reductase.

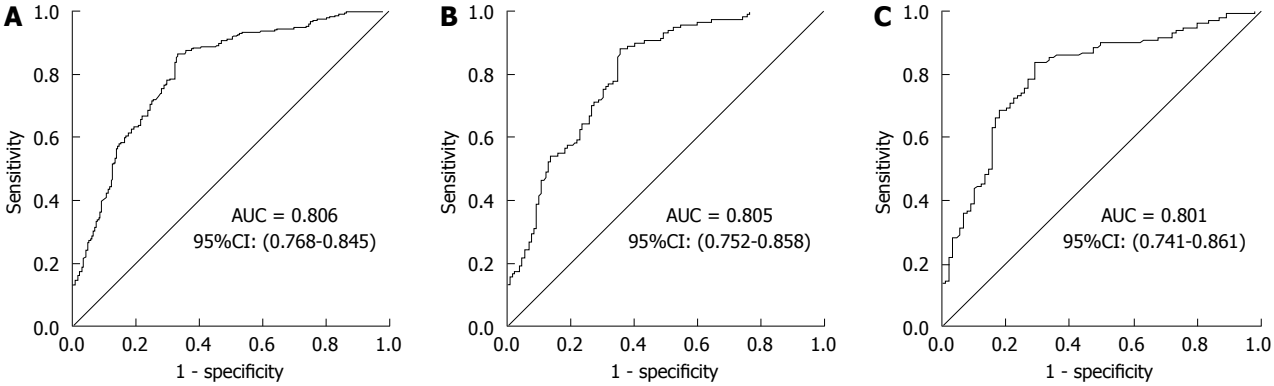

Figure 2 Receiver operating characteristic curve for serum catalase antibody. A: All subjects; B: *H. pylori*-positive subjects; C: *H. pylori*-negative subjects. *H. pylori*: *Helicobacter pylori*.

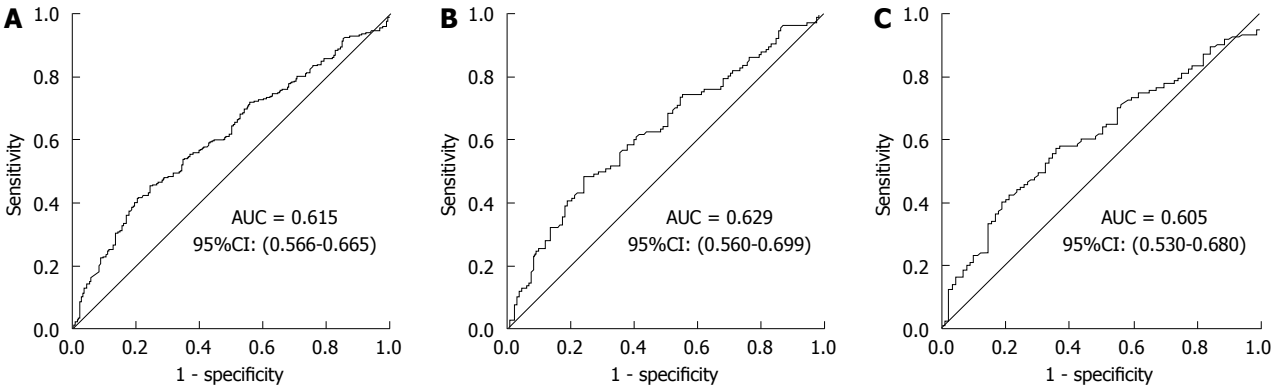

Figure 3 Receiver operating characteristic curve for serum alkyl hydroperoxide reductase antibody. A: All subjects; B: *H. pylori*-positive subjects; C: *H. pylori*-negative subjects. *H. pylori*: *Helicobacter pylori*.

AhpC could serve as biomarkers for GC, ROC curves were plotted to evaluate the screening value of the antibodies. The results showed that the AUC for KatA was 0.806, which was higher than the general standard for diagnosis (AUC $\geqslant$ 0.7)[31,32]. Unfortunately, the AUC for AhpC was lower. Generally, a single indicator for screening has a lower screening yield. At this point, we attempted to develop a combined analysis to assess the value of screening. Our previous study found that the sensitivity was 74.1%, and the specificity was 64.4%, while FlaA served as a screening biomarker for GC alone[17]. The combined results for KatA, FlaA, and AhpC showed that the AUC for combination of KatA and FlaA was elevated by 0.10, and sensitivity and specificity

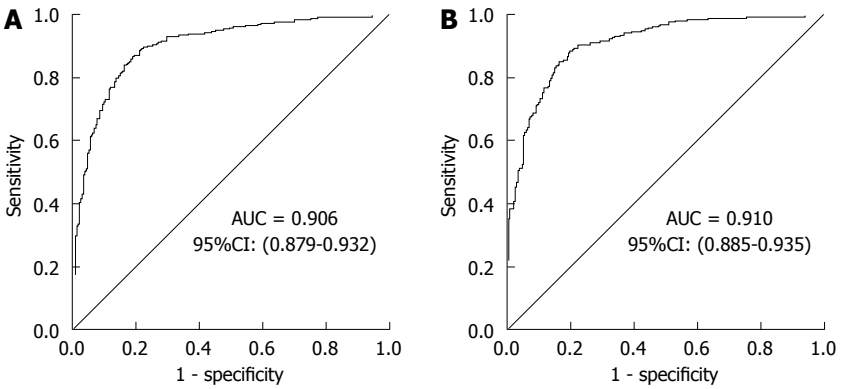

Figure 4 Receiver operating characteristic curve for combined analysis in all subjects. A: KatA + FlaA; B: KatA + FlaA + AhpC. KatA: Catalase; AhpC: Alkyl hydroperoxide reductase; FlaA: Flagella protein A.

were increased by 12.07% and 2.66%, respectively, in all subjects compared to KatA alone. Yet, combination of KatA, FlaA, and AhpC did not improve screening power in the identification of patients with GC compared to combination of KatA and FlaA.

Indirect ELISA method was adopted to detect serum KatA and AhpC antibodies in this study, and this method might be accompanied by the non-specific signal caused by cross-reactivity. In other words, KatA and AhpC will not only react with the corresponding specific antibody but also with the non-specific antibodies in the present study, Because of this non-specific signal, some *H. pylori*-negative subjects were classified as KatA or AhpC positive.

Some evidence indicates that *H. pylori* infection increases the risk of non-cardia GC[7,33]. Nine (3.88%) cardia GC cases were included in our study. However, their involvement did not affect the overall results and conclusion.

In conclusion, the data indicate that serum KatA and AhpC antibodies are associated with GC risk and that KatA may serve as a novel biomarker for GC screening. Combined analysis of KatA and FlaA could improve screening accuracy. However, serum AhpC antibody performed poorly as a marker for GC. Our study offers a basis for early diagnosis of GC, and further prospective studies are needed to verify our findings.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) infection is a crucial cause of gastric cancer (GC). Eradication of *H. pylori* seems a reasonable approach for preventing GC, but it is not feasible in large populations due to financial limitations. Therefore, a sensitive and low-cost screening biomarker for GC is urgently needed.

Research frontiers

Invasive endoscopy is the gold standard for GC detection, but it is not suitable for population-based screening. Serological testing is a widely available and noninvasive diagnostic method. In this study, the authors explored the value of serum catalase (KatA) and alkyl hydroperoxide reductase (AhpC) antibodies of *H. pylori* as biomarkers for GC monitoring.

Innovations and breakthroughs

This study indicated that KatA and AhpC antibodies are associated with GC risk and that KatA may serve as a novel biomarker for GC screening. Besides, combining for KatA and flagella protein A could improve screening accuracy.

Applications

These finding offers a basis for early diagnosis of GC.

Peer-review

This is a well-designed study showing that KatA and AhpC antibodies are associated with GC. The methodology is well described. Exploration of KatA and AhpC as biomarkers has important value for GC prevention.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Endoscopy-based management decreases the risk of postoperative recurrences in Crohn's disease

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Author contributions: All the authors equally contributed to this paper.

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Informed consent statement: The study was performed in accordance with the Declaration of Helsinki, Good Clinical Practice and applicable regulatory requirements.

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Data sharing statement: The original anonymous dataset is available on request from the corresponding author at a_buisson@hotmail.fr.

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Abstract

AIM: To investigate whether an endoscopy-based management could prevent the long-term risk of postoperative recurrence.

METHODS: From the pathology department database, we retrospectively retrieved the data of all the patients operated on for Crohn's disease (CD) in our center (1986-2015). Endoscopy-based management was defined as systematic postoperative colonoscopy (median time after surgery = 9.5 mo) in patients with

no clinical postoperative recurrence at the time of endoscopy.

RESULTS: From 205 patients who underwent surgery, 161 patients (follow-up > 6 mo) were included. Endoscopic postoperative recurrence occurred in 67.6%, 79.7%, and 95.5% of the patients, respectively 5, 10 and 20 years after surgery. The rate of clinical postoperative recurrence was 61.4%, 75.9%, and 92.5% at 5, 10 and 20 years, respectively. The rate of surgical postoperative recurrence was 19.0%, 38.9% and 64.7%, respectively, 5, 10 and 20 years after surgery. In multivariate analysis, previous intestinal resection, prior exposure to anti-TNF therapy before surgery, and fistulizing phenotype (B3) were postoperative risk factors. Previous perianal abscess/fistula (other perianal lesions excluded), were predictive of only symptomatic recurrence. In multivariate analysis, an endoscopy-based management (n = 49/161) prevented clinical (HR = 0.4, 95%CI: 0.25-0.66, P < 0.001) and surgical postoperative recurrence (HR = 0.30, 95%CI: 0.13-0.70, P = 0.006).

CONCLUSION: Endoscopy-based management should be recommended in all CD patients within the first year after surgery as it highly decreases the long-term risk of clinical recurrence and reoperation.

Key words: Crohn's disease; Postoperative recurrence; Endoscopy; Prevalence; Risk factors

Core tip: Although often recommended, the impact of an endoscopy-based management following surgery remains poorly investigated in Crohn's patients. We aimed to investigate whether an endoscopy-based management could prevent the long-term risk of postoperative recurrence in Crohn's disease (CD). We retrospectively retrieved the data of 161 patients operated on for CD in our center. We showed for the first time, that an endoscopy-based management decreased the long-term risk of clinical and surgical postoperative recurrence in CD and the risk of reoperation.

Boucher AL, Pereira B, Decousus S, Goutte M, Goutorbe F, Dubois A, Gagniere J, Borderon C, Joubert J, Pezet D, Dapoigny M, Déchelotte PJ, Bommelaer G, Buisson A. Endoscopy-based management decreases the risk of postoperative recurrences in Crohn's disease. *World J Gastroenterol* 2016; 22(21): 5068-5078 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i21/5068.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i21.5068

INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory bowel disease (IBD) of unknown etiology and can lead to digestive damage[1,2]. In the era of biologics, surgery still remains required in half of the patients ten years after diagnosis, especially in complicated diseases i.e. stenosis, abscess or fistula[3]. Surgical resection is unfortunately not curative in CD, and postoperative recurrence (POR) remains a crucial issue in these patients. The risk of reoperation is very heterogeneous in the medical literature due to different studies periods and designs, but ranges from 12% to 57% 10 years after surgery[4-7]. While clinical POR occurred in approximately half of the patients 10 years after surgery[8], three quarters (48%-93%) of patients experienced endoscopic POR within one year after surgery in referral centers[8-20].

More than 25 years ago, Rutgeerts *et al*[12] underlined that postoperative history of CD is very heterogeneous and highlighted the need to identify predictive factors of recurrence to stratify CD patients in order to optimize the therapeutic management in the immediate postoperative period. Several factors have been proposed as POR predictors (smoking, perianal lesions, previous intestinal resection, fistulizing phenotype and resection length > 50 cm), but their impact remains still debated[8,21].

Performing an endoscopy within the first year after surgery is often recommended in clinical practice[21,22]. However, the level of evidence suggesting the efficacy of such strategy remains low. Two retrospective studies reported no impact of an endoscopy-based management (EBM)[23,24]. A French group suggested, in a retrospective cohort, that an EBM was associated with a decrease risk of clinical POR at 5 years[25]. Recently, the landmark POCER trial showed that an early EBM decreased the risk of endoscopic POR at 18 mo post-surgery[26]. However the long-term impact of EBM on the risks of clinical and surgical POR remains unknown.

In the present study, we aimed to investigate whether an EBM could prevent the long-term risk of POR in CD. In addition, we aimed to report the prevalence and the risk factors of endoscopic, clinical and surgical POR, in our cohort, between 1986 and 2015.

MATERIALS AND METHODS

Ethical considerations

The study was performed in accordance with the Declaration of Helsinki, Good Clinical Practice and applicable regulatory requirements. The study was approved by local Ethics Committee (IRB number 00008526 - 2014/CE86).

Patients

We performed a retrospective study of a single-center cohort in which standardized evaluation was completed by experienced clinicians in all patients.

Table 1 Baseline characteristics of the 161 included Crohn's disease patients at the time of surgery *n* (%)

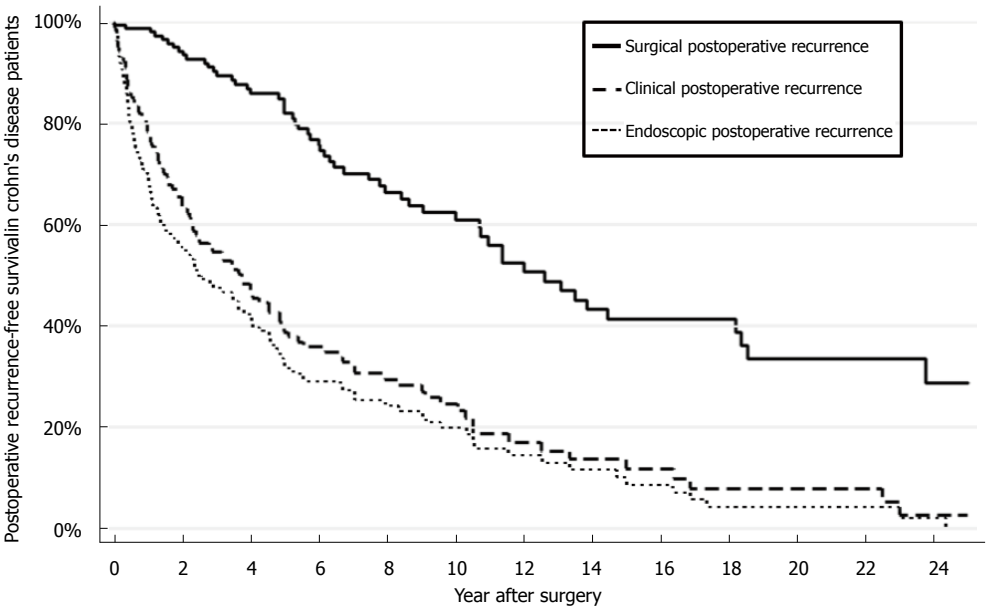
Mean age at the time of surgery (yr)	36.4 ± 13.4	Adalimumab	22 (13.7)
Mean age at diagnosis (yr)	28.7 ± 13.1	Anti-TNF naive at the time of surgery	37 (23.0)
Median disease duration (yr) (IQR)	5.8 (2.0–11.7)	Type of surgery	
Female gender	93 (57.8)	Ileocecal resection	76 (47.2)
Mean weight (kg)	60.2 ± 14.8	Ileal resection	21 (13.1)
Mean body mass index (kg/m²)	21.5 ± 4.9	Ileo-colectomy	14 (8.7)
Active smoker	53 (32.9)	Partial colectomy	31 (19.2)
Familial history of IBD	20 (12.4)	Subtotal colectomy	8 (5.0)
Previous appendectomy	67 (41.6)	Total colectomy	9 (5.6)
Previous intestinal resection	50 (31.1)	Abdomino-perianal amputation	2 (1.2)
Montreal classification		Site of anastomosis	
Age at diagnosis		Ileo-colic	91 (66.4)
A1	15 (9.3)	Ileo-rectal	9 (6.6)
A2	116 (72.1)	Ileo-ileal	21 (15.3)
A3	28 (17.4)	Colo-colonic	31 (22.6)
Crohn's disease location		Colo-rectal	7 (5.1)
L1	64 (39.8)	Stomia	
L2	21 (13.0)	None	113 (70.2)
L3	75 (46.6)	Transitory	39 (24.2)
L4	18 (11.2)	Definitive	9 (5.6)
Crohn's disease behaviour		Surgical technic of anastomosis	
B1	12 (7.4)	Stapled	46 (43.8)
B2	75 (46.6)	Handsewn	59 (56.2)
B3	74 (46.0)	Type of anastomosis	
Perianal lesions	69 (42.8)	Side-to-end	18 (18.0)
Anal ulceration, fissure	15 (9.3)	Side-to-side	54 (54.0)
Fistula/abscess	54 (33.5)	End-to-end	28 (28.0)
Medication at the time of surgery		Mean length of ileal resection (cm)	18.1 ± 17.1
5-ASA	24 (14.9)	Mean length of colonic resection (cm)	14.3 ± 17.7
Steroids	38 (23.6)	Mean length of digestive resection (cm)	31.6 ± 18.8
Budesonide	9 (5.6)	Perioperative complications	25 (16.8)
Thiopurines	36 (22.4)	Free margin resection	21 (17.1)
Methotrexate	5 (3.1)	Granuloma	47 (40.5)
Infliximab	15 (9.3)	Median CRP level, mg/L (IQR)	17.0 (3.8-61.0)

IQR: Interquartile; CRP: C-reactive protein; TNF: Tumor necrosis factor; IBD: Inflammatory bowel disease.

From the electronic database of the Pathology Department of the University Hospital Estaing of Clermont-Ferrand, France, we identified 205 patients who underwent an intestinal resection for CD, between 1986 and 2015, at the Institution. Only CD patients with a follow-up of at least 6 mo were considered for the study. Clinical, biological, pathological and endoscopic data were retrospectively collected from medical records (Table 1). As we aimed to be close to the real-life practice, we chose to include all the types of intestinal resection including patients with a definitive ostomy. For the patients with a temporary ostomy, the time point zero was defined as the time of the intestinal resection since we aimed to investigate all the potential factors influencing the time to recur (including type of resection and the presence of temporary or definitive ostomy). Surgical recurrence was defined as reoperation for CD. Clinical POR was defined, according to De Cruz *et al*[23], as recurrence of symptoms leading to hospitalization or therapeutic modifications after exclusion of other causes of recurrent symptoms such as bile-salt diarrhea, bacterial overgrowth and adhesion-related obstruction. Endoscopic POR was defined as Rutgeerts' score $\geqslant$ i2[12]. Regarding the endoscopies performed before the widespread of Rutgeerts' score use or with no score specified on the colonoscopy report, the score was evaluated retrospectively based on the content of the colonoscopy report. Patients underwent colonoscopies at their physician's discretion to assess potential subclinical disease. Patients were classified in endoscopy-based management (EBM) group if they underwent a systematic colonoscopy with no clinical POR at the time of endoscopy. All the patients included in the EBM group had a "step-up" therapeutic strategy in case of endoscopic POR[22]. The impact of the postoperative treatments was investigated in considering three groups: therapies to prevent endoscopic POR (treatment received during the period ranging from intestinal resection to endoscopic POR), therapies to prevent clinical POR (treatment received during the period ranging from endoscopic POR to clinical POR), and therapies to prevent surgical POR (treatment received during the period ranging from clinical POR to re-operation).

Data management and statistical analysis

Study data were collected and managed using REDCap electronic data capture tools hosted at Clermont-Ferrand University Hospital[27]. REDCap (Research



	1 yr	5 yr	10 yr	15 yr	20 yr
Surgical POR (%)	1.3	19.0	38.9	57.7	64.7
Clinical POR (%)	21.5	61.4	75.9	88.7	92.5
Endoscopic POR (%)	31.7	67.6	79.7	91.1	95.5

Figure 1 Kaplan Meir curves representing the prevalence of surgical, clinical and endoscopic postoperative recurrence in Crohn's disease patients undergoing intestinal resection in the Clermont-Ferrand inflammatory bowel disease unit (1986-2015).

Electronic Data Capture) is a secure, web-based application designed to support data capture for research studies, providing (1) an intuitive interface for validated data entry; (2) audit trails for tracking data manipulation and export procedures; (3) automated export procedures for seamless data downloads to common statistical packages; and (4) procedures for importing data from external sources.

Statistical analysis was performed using Stata 13 software (StataCorp LP, College Station, TX, United States). The tests were two-sided, with a type I error set at a = 0.05. Subject's characteristics were presented as mean ± SD or median (interquartile range) for continuous data (assumption of normality assessed using the Shapiro-Wilk test) and as the number of patients and associated percentages for categorical parameters. Comparisons between the independent groups were performed using the χ^2 or Fisher's exact tests for categorical variables, and using Student *t*-test or Mann-Whitney test for quantitative parameters (normality, assumption of homoscedasticity studied using Fisher-Snedecor test). Concerning the censored data, estimates were constructed using the Kaplan-Meier method. The log-rank test was used in a univariate analysis to test the prognostic value of patient characteristics for the occurrence of an event. Cox proportional hazards regression was used to investigate prognostic factors in a multivariate situation by backward and forward stepwise analysis of the factors considered significant in univariate analysis (entered into the model if $P < 0.10$) and according to clinically relevant parameters. The proportional hazard hypotheses were verified using Schoenfeld's test and plotting residuals. The interactions between possible predictive factors were also tested. Results were expressed as HRs and 95%CI.

RESULTS

Baseline characteristics of the patients

Overall, 161 CD patients were included in the study. The characteristics of these patients at the time of surgery are given in Table 1.

Prevalence of surgical, clinical and endoscopic POR

We observed a prevalence of endoscopic POR of 31.7%, 67.6%, 79.7%, 91.1% and 95.5%, respectively 1, 5, 10, 15 and 20 years after surgery (Figure 1). In our cohort, 21.5%, 61.4%, 75.9%, 88.7% and 92.5% of the patients experienced clinical POR at 1, 5, 10, 15 and 20 years, respectively (Figure 1). The rate of surgical POR was 1.3%, 19.0%, 38.9%, 57.7% and 64.7%, respectively 1, 5, 10, 15 and 20 years after surgery (Figure 1).

Risk factors of endoscopic POR

Among the 161 CD patients included in this study, 102 patients underwent a colonoscopy during their follow-up. The median interval for endoscopic POR was 2.0 years (0.6-3.6). While 54 patients (33.5%) received 5-ASA in prevention of endoscopic POR, 40 patients (24.8%), 7 patients (4.3%) and 41 patients (25.5%)

Table 2 Univariate analysis of risk factors for endoscopic postoperative recurrence in Crohn's disease

	Median time to endoscopic POR (mo)	HR [95%CI]	*P* value
Age		1.00 [0.99-1.00]	0.2
Age			
< 35 yr	41.4	Reference	
⩾ 35 yr	24.0	1.26 [0.86-1.84]	0.23
Age at diagnosis			
⩽ 16 yr	38.1	Reference	
16-40 yr	34.6	0.88 [0.47-1.67]	0.71
⩾ 40 yr	17.6	1.41 [0.60-2.63]	0.53
Tobacco use			
Non-smoker	38.1	Reference	
Active smoker	27.9	1.28 [0.77-1.70]	0.49
Previous intestinal resection			
No	43.5	Reference	
Yes	20.5	1.22 [0.98-2.15]	0.06
Total resection length > 50 cm			
No	20.5	Reference	
Yes	30.2	0.98 [0.56-1.73]	0.7
Disease behavior (Montreal classification)			
B1	-	Reference	
B2	43.5	1.30 [0.46-3.75]	0.62
B3	22.6	1.34 [0.47-3.80]	0.58
Fistulizing Crohn's disease (B3)			
No	29.3	Reference	
Yes	22.6	1.06 [0.73-1.53]	0.75
Perianal lesions			
No	34.6	Reference	
Yes	19.2	1.18 [0.82-1.71]	0.37
Type of perianal lesions			
Non-fistulizing lesions	33.4	Reference	
Fistula, abscess	20.5	1.10 [0.75-1.60]	0.23
Disease duration		1.00 [0.99-1.01]	0.77
Ileal resection > 50 cm			
No	25.9	Reference	
Yes	114.5	0.58 [0.21-1.60]	0.29
Prior exposure to anti-TNF therapy before surgery			
No	41.5	Reference	
Yes	8.0	3.91 [1.80-5.90]	< 0.001
Thiopurines therapy in prevention of endoscopic postoperative recurrence			
No	43.5	Reference	
Yes	43.7	1.07 [0.69-1.65]	0.75
Anti-TNF therapy in prevention of endoscopic postoperative recurrence			
No	41.4	Reference	
Yes	20.5	1.28 [0.78-2.13]	0.55
Period of surgery			
1986-1999		Reference	
2000-2015		1.00 [0.54-1.84]	0.99

CRP: C-reactive protein; TNF: Tumor necrosis factor.

were treated with thiopurines, methotrexate and anti-TNF, respectively. The postoperative endoscopic evaluation highlighted the following distribution: 19 patients (18.6%) classified as i0 according to the Rutgeerts' score[12], 19 patients (18.6%) as i1, 17 patients (16.7%) as i2, 12 patients (11.8%) as i3 and 35 patients (34.3%) as i4. In univariate analysis, prior intestinal resection, prior exposure to anti-TNF therapy before surgery seemed to be associated with shorter time until endoscopic POR (20.5 mo *vs* 43.5 mo, $P = 0.06$) and (8.0 mo *vs* 41.5 mo, $P < 0.001$), respectively (Table 2). Patients operated during the 1986-1999 period experienced earlier endoscopic POR than those operated during the 2000-2015 period ($P = 0.004$). In multivariate analysis, prior exposure to anti-TNF therapy before surgery (HR = 2.55, 95%CI: 1.37-4.73) and undergoing surgery during the 1986-1999 period (HR = 1.61, 95%CI: 1.04-2.49) were predictive of endoscopic POR.

Risk factors of clinical POR

Among the 161 included patients, the median time to clinical POR was 2.5 years (0.7-4.9). While 54 patients (33.5%) were treated with 5-ASA in prevention of clinical POR, 34 patients (21.1%), 2 patients (1.2%) and 26 patients (16.1%) were treated with thiopurines, methotrexate and anti-TNF, respectively. In univariate analysis, we reported that previous intestinal resection

Table 3 Univariate analysis of risk factors for clinical postoperative recurrence in Crohn's disease

	Median time to clinical POR (mo)	HR [95%CI]	*P* value
Age		1.00 [0.99-1.01]	0.4
Age			
< 35 yr	45.2	Reference	
⩾ 35 yr	30.2	1.25 [0.84-1.85]	0.27
Age at diagnosis			
⩽ 16 yr	38.1	Reference	
16-40 yr	48.0	0.81 [0.43-1.54]	0.52
⩾ 40 yr	30.2	1.03 [0.48-2.23]	0.93
Tobacco use			
Non-smoker	45.2	Reference	
Active smoker	43.7	1.00 [0.66-1.53]	0.98
Previous intestinal resection			
No	51.0	Reference	
Yes	26.6	1.62 [1.07-2.44]	0.02
Total resection length > 50 cm			
No	38.1	Reference	
Yes	33.4	1.20 [0.66-2.16]	0.55
Disease behavior (Montreal classification)			
B1	84.5	Reference	
B2	54.5	1.39 [0.48-4.00]	0.53
B3	28.9	1.61 [0.56-4.56]	0.37
Fistulizing Crohn's disease (B3)			
No	58.2	Reference	
Yes	28.9	1.21 [0.81-1.78]	0.34
Perianal lesions			
No	54.5	Reference	
Yes	26.9	1.26 [0.85-1.86]	0.24
Type of perianal lesions			
Non-fistulizing lesions	54.5	Reference	
Fistula, abscess	20.5	1.46 [1.01-2.16]	0.05
Disease duration		1.00 [0.99-1.01]	0.31
Ileal resection > 50 cm			
No	33.4	Reference	
Yes	59.5	0.72 [0.26-2.02]	0.54
Prior exposure to anti-TNF therapy before surgery			
No	48.0	Reference	
Yes	24.0	2.64 [1.24-4.33]	0.007
Thiopurines therapy in prevention of clinical postoperative recurrence			
No	44.8	Reference	
Yes	43.7	1.14 [0.74-1.76]	0.53
Anti-TNF therapy in prevention of clinical postoperative recurrence			
No	48.6	Reference	
Yes	29.3	1.39 [0.88-2.20]	0.16
Period of surgery			
1986-1999		Reference	
2000-2015		1.71 [1.12-2.63]	0.013

CRP: C-reactive protein; TNF: Tumor necrosis factor.

(51.0 mo *vs* 26.6 mo, *P* = 0.02), previous perianal fistula or abscess (54.5 mo *vs* 20.5 mo, *P* = 0.049) and prior exposure to anti-TNF therapy before surgery (24.0 *vs* 48.0, *P* = 0.007) were risk factors regarding clinical POR (Table 3). Patients operated during the 1986-1999 period experienced also earlier endoscopic POR than those operated during the 2000-2015 period (*P* = 0.013). In contrast, age at the time of surgery, age at the time of diagnosis, disease duration, tobacco use, resection length, CD behavior according to Montreal classification and all the other studied factors were not associated to an increased risk to experience clinical POR, in our cohort (Table 3). In addition, neither the use of thiopurines nor the use anti-TNF was protective factor of clinical POR. In multivariate analysis, previous intestinal resection (HR = 1.62, 95%CI: 1.07-2.46, *P* = 0.02), previous perianal abscess or fistula (HR = 1.50, 95%CI: 1.01-2.24, *P* = 0.042) and prior exposure to anti-TNF therapy before surgery (HR = 1.91, 95%CI: 1.01-3.66, *P* = 0.049) were predictive of clinical POR.

Risk factors of surgical POR

In our cohort (*n* = 161), the median time to surgical POR was 5.2 years (2.0-10.3). The medications used between the time of surgery and surgical POR were 5-ASA in 62 patients (38.5%), steroids in 78 patients (48.4%), thiopurines in 59 patients (36.6%) and anti-

Table 4 Univariate analysis of risk factors for surgical postoperative recurrence in Crohn's disease

	Median time to surgical POR (mo)	HR [95%CI]	*P* value
Age		1.00 [0.99-1.01]	0.29
Age			
< 35 yr	218.3	Reference	
⩾ 35 yr	131.6	1.32 [0.77-2.26]	0.30
Age at diagnosis			
⩽ 16 yr		Reference	
16-40 yr	144.0	1.37 [0.42-4.42]	0.60
⩾ 40 yr	108.6	1.73 [0.45-6.54]	0.42
Tobacco use			
Non-smoker	136.2	Reference	
Active smoker	173.4	0.84 [0.46-1.52]	0.56
Previous intestinal resection			
No	173.4	Reference	
Yes	108.6	1.74 [1.01-3.00]	0.04
Total resection length > 50 cm			
No	136.2	Reference	
Yes	120.0	1.50 [0.67-3.34]	0.32
Disease behavior (Montreal classification)			
B1		Reference	
B2	162.1	3.93 [0.52-29.33]	0.18
B3	128.8	5.71 [0.77-42.23]	0.09
Fistulizing Crohn's disease (B3)			
No	165.4	Reference	
Yes	128.8	1.78 [1.04-3.05]	0.03
Perianal lesions			
No	136.2	Reference	
Yes	151.1	0.99 [0.58-1.69]	0.97
Type of perianal lesions			
Non-fistulizing lesions	156.9	Reference	
Fistula, abscess	144.0	1.14 [0.66-1.97]	0.63
Disease duration		1.00 [0.99-1.01]	0.67
Ileal resection > 50 cm			
No	136.2	Reference	
Yes	120.0	1.23 [0.29-5.16]	0.78
Prior exposure to anti-TNF therapy before surgery			
No	151.1	Reference	
Yes	.	1.62 [0.92-7.08]	0.07
Thiopurines therapy in prevention of surgical postoperative recurrence			
No	144.0	Reference	
Yes	151.1	0.91 [0.50-1.65]	0.75
Anti-TNF therapy in prevention of surgical postoperative recurrence			
No	156.9	Reference	
Yes	136.2	2.09 [1.14-3.81]	0.02
Period of surgery			
1986-1999		Reference	
2000-2015		1.85 [1.22-2.80]	0.004

CRP: C-reactive protein; TNF: Tumor necrosis factor.

TNF in 69 patients (42.8%). In univariate analysis, previous intestinal resection (108.6 mo *vs* 173.4 mo, *P* = 0.04), fistulizing CD (B3 according to Montreal classification) (128.8 mo *vs* 162.1 mo, *P* = 0.03), prior exposure to anti-TNF therapy before surgery (*P* = 0.07) and anti-TNF therapy after surgery (136.2 mo *vs* 156.9 mo, *P* = 0.02) were associated with shorter time until reoperation (Table 4). The other potential risk factors investigated in the study were listed in Tables 1 and 2. In multivariate analysis, fistulizing CD (B3 according to Montreal classification) (HR = 1.78, 95%CI: 1.04-3.04, *P* = 0.003) and previous intestinal resection (HR = 1.7, 95%CI: 1.00-2.72, *P* = 0.05) were predictive of surgical POR.

Impact of an endoscopic-based management on the risk of POR

Overall, 49 of the 161 patients were included in the endoscopic-based management group. The median interval between initial surgery and endoscopy was 9.5 mo (6.0-22.9) in this group, including 63.2% of the patients (31/49) having a colonoscopy within the first year. Endoscopic POR occurred in 18 patients (36.7%) in the EBM-group. All of them underwent step-up therapeutic strategy as described in Table 5. In univariate analysis, an EBM was associated with a delayed time to clinical (33.4 mo *vs* 84.5 mo) and surgical recurrence. In multivariate analysis, an EBM decreased the risk of clinical POR (HR = 0.4, 95%CI:

Table 5 Step-up strategies in patients experiencing endoscopic postoperative recurrence in the endoscopic management-based group

Number of patient	Treatment before endoscopic evaluation	Rutgeerts' score	Treatment after endoscopic evaluation
1	None	i2	AZA
2	AZA	i3	IFX
3	AZA	i4	IFX
4	AZA	i2	AZA
5	AZA	i2	AZA (increased dose)
6	5-ASA	i4	IFX
7	ADA eow	i3	ADA ew
8	None	i4	ADA
9	5-ASA	i4	IFX + MTX
10	AZA	i4	IFX
11	AZA	i2	AZA (increased dose)
12	IFX + MTX	i3	IFX (increased dose) + MTX
13	ADA eow	i4	ADA ew
14	None	i2	AZA
15	None	i3	ADA
16	None	i2	AZA
17	None	i2	AZA
18	ADA eow	i3	ADA ew

AZA: Azathioprine; MTX: Methotrexate; IFX: Infliximab; ADA: Adalimumab; eow: Every other week; ew: Every week.

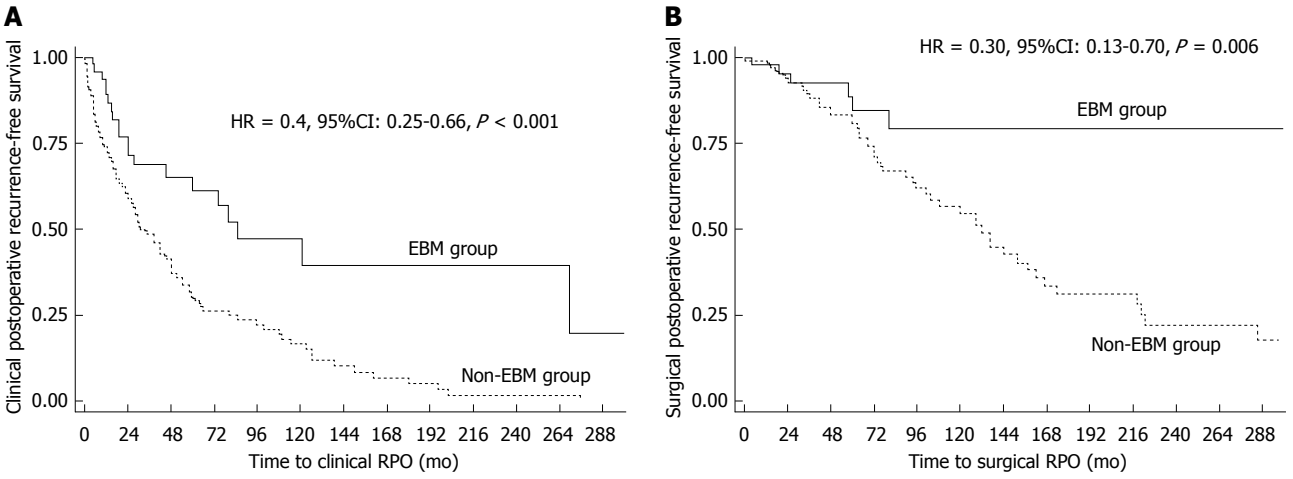


Figure 2 Long-term impact of endoscopic-based management on and clinical (A) and surgical (B) postoperative recurrence in Crohn's disease.

0.25-0.66, $P < 0.001$) (Figure 2A) and surgical POR (HR = 0.30, 95%CI: 0.13-0.70, $P = 0.006$) (Figure 2B).

DISCUSSION

Although performing a colonoscopy within the first year following surgery is commonly recommended in daily practice, the level of evidence suggesting that an EBM is an efficient strategy remains poorly investigated and is limited to short-term outcomes[23-26]. We reported here, the long-term impact of an EBM on the surgical and clinical POR risk that it has never been reported so far.

The prevalence of endoscopic POR in our cohort was perfectly in line with data from population-based cohort, which showed more than half of patients are experiencing endoscopic POR at 5 years, three quarters at 10 years and more than 90% at 15 years[3,28-30]. Our results also highlighted that more than three quarters (75.9%) of the patients experienced clinical POR within 10 years after surgery, that clinical symptoms occurred in almost all the CD patients followed in referral centers (92.5% at 20 years) and that almost two thirds (64.7%) of the CD patients were re-operated within 20 years of surgery. These data confirmed that surgery is not curative in CD in the large majority of the cases.

In our cohort, we confirmed that patients who underwent prior intestinal resection for CD, had higher risks of surgical, clinical and endoscopic POR, as previously showed in both population-based cohort[29] and referral centers[19]. In addition, we found that a fistulizing phenotype (B3 according to the Montreal classification) was associated with higher risk of endoscopic and surgical POR according to the results of a meta-analysis including 13 studies and 3044 patients (OR = 1.5, $P = 0.002$)[31] and several referral center-based studies[8]. Surprisingly, we did not show

any influence of tobacco use on the risk of POR in our cohort. However, smoking is often considered as the strongest risk factor for postoperative recurrence, increasing by twofold the risk of clinical recurrence and multiplying by 2.5 the risk for surgical POR within 10 years, with a dose-response relationship[21,32,33]. It could be partly explained by the retrospective design of our study and the fact that studying smoking habits is very difficult due to a wide modification of the smoking status during this long-term follow-up, the hardness to evaluate accurately the consumption of cigarettes and the underestimation of the number of smokers. Perianal disease is often admitted as predictor for POR. However, it remains unclear whether perianal lesions directly impacted the postoperative course of luminal disease or was only associated with perianal disease relapse leading to therapeutic modifications. In our cohort, the overall perianal lesions including both fistulizing and non-fistulizing (ulceration, fissure) lesions did not show any impact on the rate of recurrence. In contrast, we observed that prior perianal fistula or abscess was associated with increased clinical POR rate, but it did not influence the risk of both endoscopic and surgical POR. Most of the previous data indicated that perianal lesions were associated with clinical POR[28,34,35], while neither the studies investigating the risk factors for surgical POR[8,36,37], nor those interested in risk factors for endoscopic POR[8] achieved to prove the role of perianal involvement in the postoperative course of CD. Our results seemed to confirm that perianal involvement did not influence the risk of luminal recurrence, but underlined the fact that patients with perianal involvement had an increased risk of perianal symptomatic recurrence. Accordingly, we suggest that these patients require aggressive treatment after surgery, preferably to prevent perianal relapse rather than luminal recurrence, but this point warrants to be validated in additional studies. Some authors suggested using anti-TNF therapy in prevention of endoscopic POR in patients with prior exposure to anti-TNF before surgery[22]. This statement is based on experts' opinion rather than evidence-based medicine. However, in our cohort, we found that prior exposure to anti-TNF therapy before surgery is the most relevant risk factor for POR. It could mean that anti-TNF agents prescription associated to the most severe disease could predict an unfavorable postoperative course in CD. Stratifying the patients according to their risk factors of POR remains a key point in the management of the postoperative period in CD. However, the known risk factors do not allowed to accurately select the high-risk patients. Histologic factors, especially plexitis, could improve the selection of CD patients with ileocolic resection[38-41].

Although early colonoscopy after surgery is recommended in ECCO guidelines[21], low evidence supports this recommendation to date. Two retrospective studies evaluating the impact of postoperative EBM with tailored treatment according to the endoscopic findings did not report any benefit of this strategy on both clinical and surgical POR[23,24]. Bordeianou *et al*[24] reported no significant difference in time to clinical POR among the three following groups (n = 199 patients): immediate postoperative treatment, tailored treatment after endoscopy and no treatment. Similarly, De Cruz *et al*[23] reported no clinical benefit from an EBM in 136 CD patients. The authors explained their negative results in noting that the response to the endoscopic findings was not standardized and immunosuppressive therapy was uncommon during their study period. More recently, among 132 operated on for CD from the Saint-Louis Hospital, Paris, France, the authors reported a decreased clinical POR rate 5 years after surgery, in the patients with EBM, compared to the non-EBM group (26% *vs* 52%)[25]. Recently, the landmark POCER trial, a prospective, well-designed study, compared the impact of a tailored management according to clinical risk of recurrence, with early colonoscopy and treatment step-up on recurrence[26]. The results showed that an early EBM, performed 6 mo after surgery, decreased the rate of endoscopic POR at 18 mo[26]. However the long-term impact of EBM on the risk of POR (especially surgical) remained unknown. Our results indicated for the first time that an EBM influenced the risk of reoperation for CD, leading to a delayed time before surgical POR. In addition, we confirmed that the EBM group experienced less clinical POR over time than the non-EBM group, in a long period of follow-up. As our cohort overlapped a very long period with different available medications overtime, we did not show any impact of the postoperative treatment, especially biologics, in this population.

The main limits of this study were the retrospective and monocentric design. In addition, the time of endoscopy (median = 9.5 mo after surgery) and the step-up strategy were not standardized for all the patients. However, we observed for the first time the positive impact of an EBM on the risk of reoperation in CD, in a cohort monitored during almost 30 years (1986-2015) and based on a Pathology Department electronic database (consecutive patients).

In conclusion, POR is very frequent in CD and remains a critical issue in the management of the postoperative period. The identification of predictors to select the high-risk patients warranting top-down strategy in the postoperative period is crucial. An endoscopic-based management within the first year after surgery decreases the risk of symptoms recurrence and reoperation and then have to be recommended in daily practice.

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COMMENTS

Background

Surgical resection is unfortunately not curative in Crohn's disease (CD), and postoperative recurrence (POR) remains a crucial issue in these patients. Performing an endoscopy within the first year after surgery is often recommended in clinical practice. However, the level of evidence suggesting the efficacy of such strategy remains low. Two retrospective studies reported no impact of an endoscopy-based management (EBM). A French group suggested, in a retrospective cohort, that an EBM was associated with a decrease risk of clinical POR at 5 years. Recently, the landmark POCER trial showed that an early EBM decreased the risk of endoscopic POR at 18 months post-surgery. However the long-term impact of EBM on the risks of clinical and surgical POR remains unknown.

Research frontiers

The level of evidence suggesting the efficacy of an endoscopy-based strategy in CD remains low especially in the long-term. In the present study, the authors aimed to investigate whether an endoscopy-based strategy could prevent the long-term risk of POR in CD.

Innovations and breakthroughs

This paper showed for the first time, that an endoscopic-based management within the first year after surgery decreases the long-term risk of symptoms recurrence and reoperation.

Applications

Endoscopy-based management should be recommended in all CD patients within the first year after surgery in daily practice as it highly decreases the long-term risk of clinical recurrence and reoperation.

Terminology

An endoscopy-based strategy in CD means treatment intensification in case of endoscopic recurrence to prevent symptoms reappearance.

Peer-review

This article deals with an important aspect of CD- post operative recurrence. The article is well written in general.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Ulcerative colitis patients in clinical remission demonstrate correlations between fecal immunochemical test results, mucosal healing, and risk of relapse

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Author contributions: All authors contributed to this paper.

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Informed consent statement: All study participants or their legal guardians provided informed written consent regarding personal and medical data collection prior to study enrollment.

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Data sharing statement: The original anonymous dataset is available upon request from the corresponding author at sakikoh@cc.okayama-u.ac.jp.

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Abstract

AIM: To assess the risk of relapse in ulcerative colitis (UC) patients in clinical remission using mucosal status and fecal immunochemical test (FIT) results.

METHODS: The clinical outcomes of 194 UC patients in clinical remission who underwent colonoscopy were based on evaluations of Mayo endoscopic subscores (MESs) and FIT results.

RESULTS: Patients with an MES of 0 (n = 94, 48%) showed a ten-fold lower risk of relapse than those with an MES of 1-3 (n = 100, 52%) (HR = 0.10, 95%CI: 0.05-0.19). A negative FIT result (fecal hemoglobin concentrations $\leqslant$ 100 ng/mL) was predictive of patients with an MES of 0, with a sensitivity of 0.94 and a specific of 0.76. Moreover, patients with a negative FIT score had a six-fold lower risk of clinical relapse than those with a positive score (HR = 0.17, 95%CI: 0.10-0.28). Inclusion of the distinguishing parameter, sustaining clinical remission > 12 mo, resulted in an even stronger correlation between negative FIT results

and an MES of 0 with respect to the risk of clinical relapse (HR = 0.11, 95%CI: 0.04-0.23).

CONCLUSION: Negative FIT results one year or more after remission induction correlate with complete mucosal healing (MES 0) and better prognosis. Performing FIT one year after remission induction may be useful for evaluating relapse risk.

Key words: Ulcerative colitis; Clinical remission; Mucosal healing; Mayo endoscopic subscore; Quantitative fecal immunochemical test

Core tip: Mucosal healing has been recognized as the treatment goal of. In this study, the relapse rate differed greatly between patients with a Mayo endoscopic subscore (MES) of 0 and an MES of 1 such that mucosal healing should be defined as an MES of 0. We previously reported that a negative fecal immunochemical test (FIT) correlates positively with mucosal healing. This paper indicated that patients with a negative FIT demonstrated a lower risk of clinical relapse than those with a positive FIT and that the risk of relapse in patients in prolonged remission and with a negative FIT was equivalent to that of patients with an MES of 0.

Nakarai A, Kato J, Hiraoka S, Takashima S, Takei D, Inokuchi T, Sugihara Y, Takahara M, Harada K, Okada H. Ulcerative colitis patients in clinical remission demonstrate correlations between fecal immunochemical test results, mucosal healing, and risk of relapse. *World J Gastroenterol* 2016; 22(21): 5079-5087 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i21/5079.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i21.5079

INTRODUCTION

Ulcerative colitis (UC) is an idiopathic chronic inflammatory disorder that, when untreated, results in symptoms of diarrhea and bloody stool. Current studies evaluating UC treatment using colonoscopy cite a need to achieve not only clinical responses but also mucosal healing, which is associated with sustained clinical remission and reduced rates of hospitalization and surgical resection[1]. An additional study indicated that early mucosal healing after the administration of infliximab for UC correlates with improved clinical outcomes, including the avoidance of colectomy[2]. Another report showed that a lack of mucosal healing after initial corticosteroid therapy is associated with late negative outcomes[3].

Nevertheless, standardized criteria for evaluating disease severity and the degree of mucosal healing are not presently available[4]. Some reports define mucosal healing as an MES of 0 or 1[2,5], whereas other reports consider only an MES of 0 to be healing[6]. Such inconsistencies complicate interpretations of the significance of mucosal healing in the treatment of UC such that differences in long-term prognosis (evaluated by relapse of clinical symptoms and/or colectomy) between clinically asymptomatic patients with an MES of 0 (complete mucosal healing) and those with an MES of 1 (partial mucosal healing) are rarely reported.

Using colonoscopy to evaluate mucosal status in UC patients is expensive and invasive. Previous work reported by our group demonstrates that a quantitative fecal occult blood test (FIT) effectively reflects the mucosal status of patients with UC and that a negative FIT correlates strongly with mucosal healing[7]. Although we found a significantly higher positive correlation between negative FIT results and an MES of 0 (> 90%) compared with an MES of 0 or 1 (< 60%), the likelihood of relapse in patients in remission with a negative FIT has not been formally evaluated.

In this study, we retrospectively reevaluated and subdivided the colonoscopic findings of UC patients in clinical remission into subcategories of MES 0 or MES 1. Patient prognoses (relapse of clinical symptoms and colectomy rate) were evaluated to determine whether the optimal goal of UC treatment should be either an MES of 0 or 1 or only an MES of 0. Correlations between FIT results and MES in these patients were also evaluated to determine whether FIT scores can function as a surrogate marker for meeting the treatment goals of UC patients in clinical remission.

MATERIALS AND METHODS

Patients

Between January 2006 and January 2014, ambulatory UC patients who were making periodic visits to Okayama University Hospital were requested to prepare and bring fecal samples to scheduled colonoscopy appointments for an evaluation of disease activity and surveillance. Fecal samples (prior to colonoscopy bowel preparation) were tested for fecal occult blood with an FIT, and the results were evaluated with regard to colonoscopic findings. All of the patients had an established diagnosis of UC according to endoscopic and histologic assessments and had received medical therapy.

Clinical disease activity was scored using the Mayo score, which is based on the following four criteria: stool frequency, rectal bleeding, endoscopic findings, and physician global assessment (0, normal; 1, mild disease; 2, moderate disease; 3, severe disease)[8]. Clinical remission was defined as a partial Mayo score (Mayo score without endoscopic findings) $\leqslant$ 2 points, with no individual subscore exceeding 1 point[5]. Clinical relapse was defined by an increase or modification of concomitant medications due to a worsening of symptoms. Patients in clinical remission at the time of colonoscopy were considered eligible

for this retrospective cohort study. The study protocol was approved by the Institutional Review Board of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences.

Fecal sampling and instrument for FIT analysis

The details of the method used for the FIT were described previously[9-11]. Briefly, patients prepared fecal samples before bowel preparation for colonoscopy using an OC-Hemodia sampling probe (Eiken Chemical, Tokyo, Japan) provided by the manufacturer of the kit. An 8 cm × 2 cm test tube-shaped container holds the sampling probe. The patient inserts the probe into several different areas of stool and then firmly places it back into the tube for sealing. The probe tip with the fecal sample is suspended in a standard volume of hemoglobin-stabilizing buffer. Submitted stool samples were immediately processed and examined using OC-SENSOR neo (Eiken Chemical), which can accurately measure fecal hemoglobin concentrations of 50 ng/mL to 1000 ng/mL. Fecal specimens with a hemoglobin concentration over 1000 ng/mL were measured following dilution. Because FIT results are inaccurate at hemoglobin concentrations below 50 ng/mL, specimens with a hemoglobin concentration in this range were categorized as one (0-50 ng/mL).

Colonoscopy

On the day of the colonoscopy, patients received a polyethylene glycol-based or magnesium citrate-based electrolyte solution for bowel preparation and ingested it according to the manufacturer's instructions. After colonic lavage, the patients underwent colonoscopy. Patients were excluded if the colonoscopic examination was incomplete due to problems with the bowel preparation or if the colonoscope could not be inserted into the cecum. At colonoscopy, the colonoscopists were not blinded to the clinical data. However, at data collection for analysis, colonoscopic images were re-evaluated by experienced colonoscopists who did not know the clinical data.

The mucosal status of UC was assessed using the MES classification system. Evaluation was performed at each portion of the colorectum (cecum; ascending, transverse, descending and sigmoid colon; and rectum), and the maximum score in the colorectum of each patient was used for analysis. An MES of "0" throughout the colorectum was defined as complete mucosal healing, whereas a maximum MES of "1" in the colorectum was defined as partial mucosal healing.

Statistical analysis

Statistical analyses were performed using JMP version 9 (SAS Institute, Cary, NC, United States). A Kaplan-Meier curve estimating the duration of sustained clinical remission was generated for each predefined patient group, and comparisons between groups were performed using a 2-sided log-rank test. The Cox proportional hazards regression model was used to calculate hazard ratios (HRs) between groups, quantifying the likelihood of clinical relapse using a 95%CI. Comparative analyses, such as a χ^2 test and the Mann-Whitney test, were used for cross-sectional analysis of categorical data. Spearman rank correlation was performed to measure the association between fecal hemoglobin concentrations and MES, and trends between these values were evaluated using the Cochran-Armitage trend test. A receiver operating characteristic (ROC) curve was generated to estimate appropriate cutoff values for the FIT. The area under the curve (AUC), and sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV), for detecting mucosal healing based on the FIT results was calculated. All *P*-values were two-sided and considered significant when less than 0.05.

RESULTS

Clinical characteristics of the patients

A total of 248 UC patients who underwent colonoscopy between January 2006 and January 2014 also underwent a corresponding FIT. If a patient underwent two or more colonoscopies during remission, then only the data from the first colonoscopy were included. Among these patients, 194 (78%) demonstrated clinical remission at the time of colonoscopy and were enrolled in the study.

Table 1 summarizes the clinical characteristics of the 194 patients (99 male and 95 female; median age at UC onset 32 years). At the time of colonoscopy, the median duration of sustaining clinical remission was 11 mo. Colonoscopic findings revealed that the maximum MES was 0 in 94 (49%) cases, 1 in 57 (29%) cases, 2 in 39 (20%) cases, and 3 in 7 (2%) cases. On the basis of our definitions, 94 and 57 patients had complete and partial mucosal healing, respectively. Among the 194 patients, 111 (57%) cases showed fecal hemoglobin concentrations of 100 ng/mL or lower and were defined as FIT-negative.

Difference in the prognosis of UC patients according to the MES

Kaplan-Meier curves comparing the maintenance of clinical remission among patients with an MES of 0-3 are shown in Figure 1. There was a statistically significant difference in remission maintenance rates between each MES group ($P < 0.0001$, log-rank test). The Cox proportional hazards model suggested that patients with an MES of 1 were more than seven times more likely to relapse than patients with an MES of 0 (HR of MES 1 *vs* MES 0, 7.40; 95%CI: 3.78-15.06). Conversely, MES 0 patients were approximately ten times less likely to relapse than MES 1-3 patients (HR = 0.10; 95%CI: 0.05-0.19). Furthermore, the risk of colectomy or the occurrence of dysplasia/cancer did not vary significantly between patients in different

Table 1 Incidence of clinical characteristics among participants

Total	194
Gender, *n* (%)	
Male	99 (51)
Female	95 (49)
Extent of disease, *n* (%)	
Pancolitis	125 (64)
Left-side colitis	48 (25)
Proctitis	21 (11)
Median (IQR) age at onset	32 (22-43)
Median (IQR) duration of disease, months	107 (51-194)
Median (IQR) age of undergoing colonoscopy	44 (33-56)
Median (IQR) duration of sustaining clinical remission at the time of colonoscopy, months	11 (6-23)
Concomitant medications, *n* (%)	
Aminosalicylate	178 (92)
Corticosteroids	27 (14)
Mercaptopurine/Azathioprine	79 (41)
Tacrolimus	10 (5)
Biologics	9 (5)
Colonoscopy findings, *n* (%) (maximum index in the colorectum)	
MES 0	94 (49)
MES 1	57 (29)
MES 2	39 (20)
MES 3	4 (2)
Fecal Hb concentrations (ng/mL), *n* (%)	
0-100	111 (57)
101-1000	56 (29)
1001-10000	20 (10)
10001-	7 (4)

MES: Mayo Endoscopic subscore; Hb: Hemoglobin; IQR: Interquartile range.

MES groups (data not shown). Overall, these results suggest that the treatment goal for minimizing relapse in UC patients in clinical remission should be to achieve a score of MES 0, rather than MES 1.

Comparison of clinical characteristics in MES 0 patients relative to MES 1-3 patients

We have shown that achievement of complete mucosal healing (MES 0) is optimal for UC patients with regard to the maintenance of clinical remission. We also compared other clinical characteristics of the 94 patients in clinical remission with complete mucosal healing (MES 0) with those of 100 patients who showed only partial healing (MES 1) or more inflammation (MES 2, 3) (Table 2). The former subgroup had maintained clinical remission for a significantly longer time at the time of colonoscopy (17 mo *vs* 9 mo, $P < 0.0001$) and was administered mercaptopurine/azathioprine more frequently than the latter (45 patients *vs* 34 patients, $P = 0.049$). The FIT results demonstrated that fecal hemoglobin concentrations were significantly lower (50 ng/mL *vs* 315 ng/mL, $P < 0.0001$) in patients with complete mucosal healing than in patients with partial healing or more inflammation.

Applicability of FIT results for predicting complete mucosal healing in UC patients in clinical remission

Our data demonstrated that MES 0 patients in clinical remission presented significantly lower fecal hemoglobin concentrations than MES 1-3 patients. The correlation between FIT results and colonoscopic findings among these patient subgroups is illustrated in Figure 2. The Spearman rank correlation coefficient quantifying the relationship between FIT values and MES subgroups was 0.6530 ($P < 0.0001$). The proportion of cases with fecal hemoglobin concentrations ⩽ 100 ng/mL was greatest in the MES 0 patients (88/94, 92%), and decreased gradually as the MES increased (MES 1: 16/57, 28%; MES 2: 6/39, 15%). The trend of the decrease in the relationship

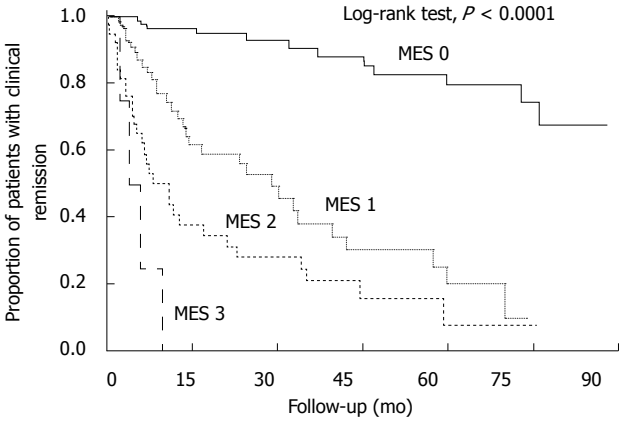

Figure 1 Kaplan-Meier curves depicting the rates of clinical remission maintenance with regard to the Mayo endoscopic subscores. There were statistically significant differences in the cumulative remission maintenance rates between patients in each Mayo endoscopic subscore (MES) subgroup ($P < 0.0001$, log-rank test). The hazard ratio for risk of relapse of patients with an MES of 0 relative to those with an MESs of 1-3 was 0.10 (95%CI: 0.05-0.19).

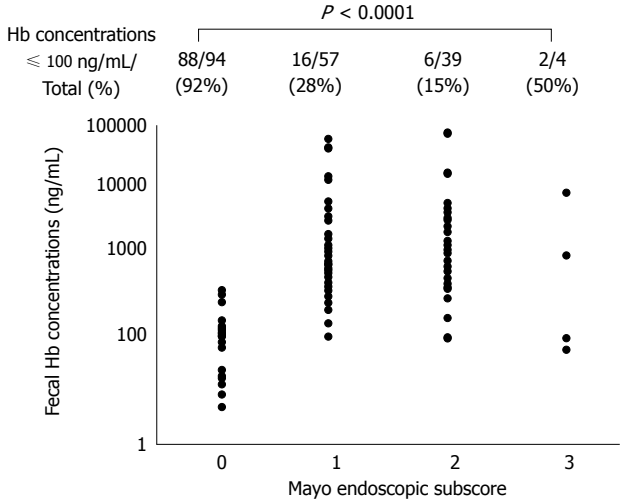

Figure 2 Correlation between fecal immunochemical test results and the Mayo endoscopic subscores. There was a significant positive correlation between fecal hemoglobin concentrations and the MES (Spearman rank correlation coefficient = 0.6530, $P < 0.0001$). The proportion of cases with negative FIT results (fecal hemoglobin concentration ⩽ 100 ng/mL) was greatest in cases with an MES of 0 (88/94, 92%). The proportion decreased gradually as the MES increased (MES 1: 16/57, 28%, MES 2: 6/39, 15%), and the trend of the decrease in relation to the MES was statistically significant ($P < 0.0001$, Cochran-Armitage trend test).

Table 2 Characteristics of patients with MES 0 *vs* MES 1-3

Characteristics	MES 0 (*n* = 94)	MES 1-3 (*n* = 100)	*P* value
Gender, *n* (%)			
Male	54 (57)	45 (45)	0.083
Female	40 (43)	55 (55)	
Median age, yr (IQR)	46 (32-60)	41 (33-51)	0.051
Median duration of disease, mo (IQR)	99 (53-198)	112 (44-191)	0.950
Median age at onset, yr (IQR)	34 (22-47)	31 (22-40)	0.130
Median duration of sustaining clinical remission at the time of colonoscopy, mo (IQR)	17 (9-33)	9 (4-13)	< 0.0001
Extent of disease, *n* (%)			
Pancolitis	59 (63)	66 (66)	0.340
Left-side colitis	27 (29)	21 (21)	
Proctitis	8 (8)	13 (13)	
Concomitant medications, *n* (%)			
Aminosalicylate	86 (91)	92 (92)	0.900
Corticosteroids	11 (12)	16 (16)	0.390
Mercaptopurine/ Azathioprine	45 (48)	34 (34)	0.049
Tacrolimus	4 (4)	6 (6)	0.580
Biologics	2 (2)	7 (7)	0.110
Fecal Hb concentrations (ng/mL)	50 (4-50)	315 (108-1277)	< 0.0001

MES: Mayo endoscopic subscore; Hb: Hemoglobin; IQR: Interquartile range.

to the MES was statistically significant ($P < 0.0001$, Cochran-Armitage trend test).

Figure 3 shows the ROC curve for fecal hemoglobin concentrations in relation to complete mucosal healing. A cutoff value of 100 ng/mL was shown to effectively differentiate between patients with and without complete mucosal healing at a sensitivity of 0.94 and a specificity of 0.76. The PPV of applying 100 ng/mL as a cut-off for determining complete mucosal healing was 0.79, whereas the NPV was 0.93. The corresponding AUC was 0.88.

In addition to the results relating FIT to mucosal healing, Kaplan-Meier curves showed that the cumulative remission maintenance rate was also significantly different between patients with fecal hemoglobin concentrations ⩽ 100 ng/mL and those with fecal hemoglobin concentrations > 100 ng/mL ($P < 0.0001$, log-rank test; Figure 4). The Cox proportional hazards model indicated that patients with a negative FIT value had a six-fold lower risk of clinical relapse than those with a positive FIT (HR = 0.17; 95%CI: 0.10-0.28).

Differences between MES 0 and MES 1-3 FIT negative patients

In UC patients in clinical remission, negative FIT values correlate closely with an MES of 0, and patients in either clinical category demonstrate a better prognosis. However, as a marker for a reduced risk of UC relapse, a rating of MES 0 is slightly more accurate in predicting risk than a negative FIT value (HR = 0.10 *vs* 0.17). In a comparison between Kaplan-Meier curves of MES

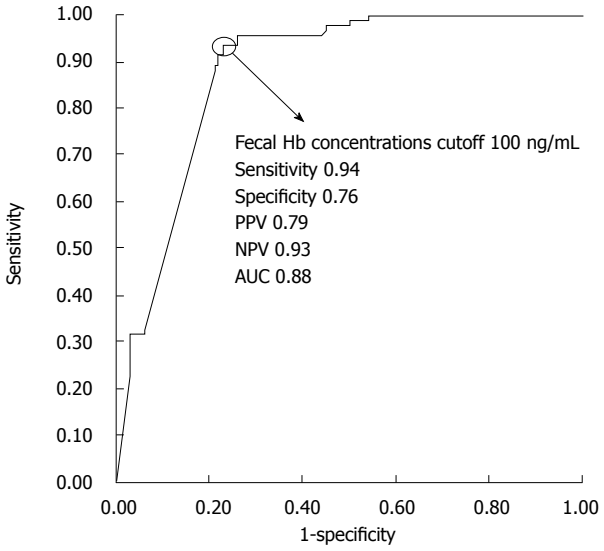

Figure 3 Receiver operating characteristic curve of fecal hemoglobin concentrations for predicting complete mucosal healing. A cutoff value of 100 ng/mL differentiated between patients with or without complete mucosal healing with the following values: 0.94 sensitivity, 0.76 specificity, 0.79 PPV, 0.93 NPV, and 0.88 accuracy. The corresponding area under the curve was 0.88.

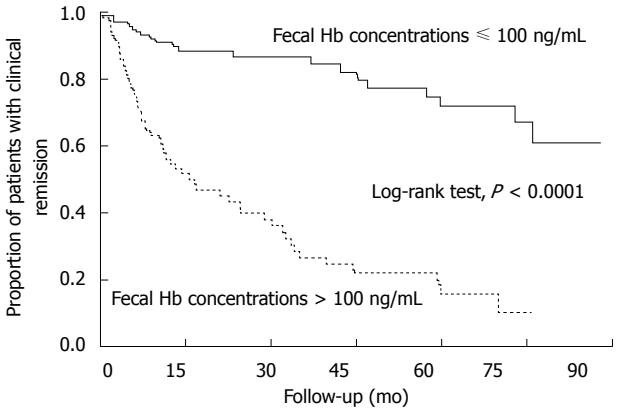

Figure 4 Kaplan-Meier curves depicting maintenance of clinical remission with regard to fecal immunochemical test results. There was a statistically significant difference in cumulative remission maintenance rates between the patients with a negative FIT result and those with a positive FIT result ($P < 0.0001$, log-rank test). The hazard ratio relating the relapse risk in patients with a negative FIT to those with a positive FIT was 0.17 (95%CI: 0.10-0.28).

0 (Figure 1) and negative FIT (Figure 4), the relapse rate within one year after colonoscopy/FIT was slightly higher in patients with a negative FIT than in those with an MES of 0 (1 year relapse rate 9% *vs* 3%, and 5 year relapse rate 22% *vs* 17%, respectively). In addition, the duration of sustaining clinical remission at colonoscopy/FIT was significantly longer in patients with an MES of 0 than in those with an MES of 1-3 among patients with a negative FIT (16 mo *vs* 8 mo, $P = 0.001$). These findings suggest that patients who enter clinical remission are more likely to demonstrate a negative FIT first (cessation of colorectal bleeding), followed by complete mucosal healing.

Because the MES 0 patients sustained clinical

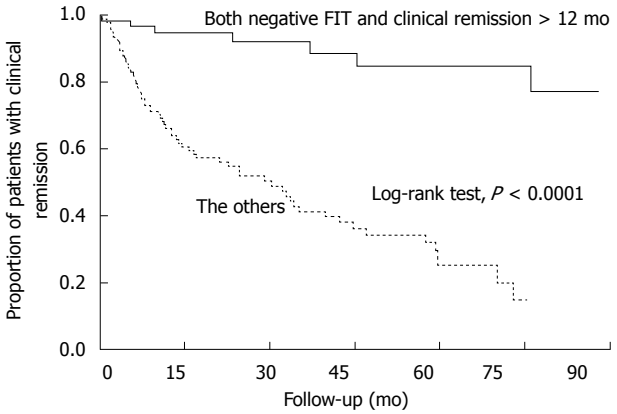

Figure 5 Kaplan-Meier curves depicting the maintenance of clinical remission in patients with both a negative fecal immunochemical test and clinical remission > 12 mo relative to all other patients. There was a statistically significant difference in the cumulative remission maintenance rates between patients with both a negative FIT and clinical remission > 12 mo (n = 66) and all other patients (n = 128) (P < 0.0001, log-rank test). The hazard ratio relating relapse risk in patients with both a negative FIT and clinical remission > 12 mo to relapse risk in all other patients was 0.11 (95%CI: 0.04-0.23).

remission for a longer time than the MES 1-3 patients, we performed multivariate analysis and found that clinical remission > 12 mo was a significant factor for predicting an MES of 0 among our 194 subjects (OR = 8.47; 95%CI: 3.33-24.03). Thus, we used Kaplan-Meier curves to illustrate the relationship between the patients who fulfilled both classifiers - negative FIT and clinical remission > 12 mo - and all others (Figure 5). The Cox proportional hazards model indicated that patients with both a negative FIT and clinical remission > 12 mo have a nine-fold lower risk of relapse than all other patients (HR = 0.11, 95%CI: 0.04-0.23). The one-year and five-year relapse rates for this group (FIT negative, remission > 12 mo) were similar to those of patients with MES 0 (4% and 15%, respectively). Taken together, these findings suggest that a negative FIT in patients who have sustained clinical remission for at least one year is a good indicator of complete mucosal healing and a predictor of low relapse risk.

Figure 6 indicated the proposed workflow of the follow-up of UC patients using FIT. During remission induction therapy, we recommend to measure FIT about once 2-4 wk, comparing those results to the baseline FIT result. When FIT results decrease, we make therapy maintained or weakened. On the other hand, when FIT results do not decrease or increase, therapy should be considered to intensify. After remission induction, we recommend to measure FIT every visit. Since patients with both negative FIT and clinical remission > 12 mo are highly probable to have achieved mucosal healing with low risk of relapse, these patients could be followed with longer intervals. Otherwise, patients are considered to have residual inflammation with considerable risk of relapse, they need to be followed up closely.

DISCUSSION

In this study, our goal was to distinguish which MES scores represent optimal mucosal healing - an MES of 0 alone or an MES of 0 or 1. In addition, because we previously reported that FIT can function as a predictor of mucosal status in UC patients, we further discriminated the predictability of the FIT as a measure of mucosal status and of the prognosis of patients with clinical remission. In retrospective analyses of the differences in long-term clinical outcomes between patients with an MES of 0 and those with an MES 1, we found that negative FIT results were significantly more likely in patients with complete mucosal healing (MES 0) than in patients with partial mucosal healing (MES 1). Our findings showed that patients with an MES of 0 alone were much less likely to relapse and that negative FIT results showed a stronger positive correlation with an MES of 0 alone than with an MES of 0 or 1, as well as with a reduced risk of relapse. Moreover, analyses including the parameter "sustaining clinical remission > 12 mo" revealed a more robust correlation between negative FIT results and complete mucosal healing with regard to the minimum risk of relapse.

Standardized criteria for evaluating the severity of ulcerative colitis and for defining mucosal healing in patients with UC have yet to be established[4]. Many prior clinical studies have defined mucosal healing as maintaining an MES of 0 or 1[2,5], and there are few long-term studies distinguishing the ability of an MES of 0 or an MES of 1 to contribute to the maintenance of clinical remission in UC patients. Detailed analysis of findings from the Active Ulcerative Colitis Trials (ACT-1, 2)[2] showed that the clinical remission rate at 54 wk from the time of colonoscopy (performed after induction of remission at week 8) was 73% in MES 0 patients and 47% in MES 1 patients; more than half of the MES 1 patients relapsed. In addition, other groups including our group, reported that patients in clinical remission with an MES of 0 were less likely to relapse than those with an MES of 1 or more, using a retrospective cohort[12,13]. In contrast, in a study evaluating the effectiveness of mesalazine for maintaining UC remission, Meucci *et al*[6] reported no significant difference between the rates of relapse at one year in MES 0 *vs* MES 1 patients.

Negative FIT results among the subjects in clinical remission also correlated closely with an MES of 0, and the patients in clinical remission who showed a negative FIT result were less likely to relapse than those with a positive FIT. These results suggest that a negative FIT result may function as a surrogate marker for complete mucosal healing and should be the treatment goal for UC patients in remission.

Nevertheless, our data do not show a complete overlap between the clinical behavior of patients with

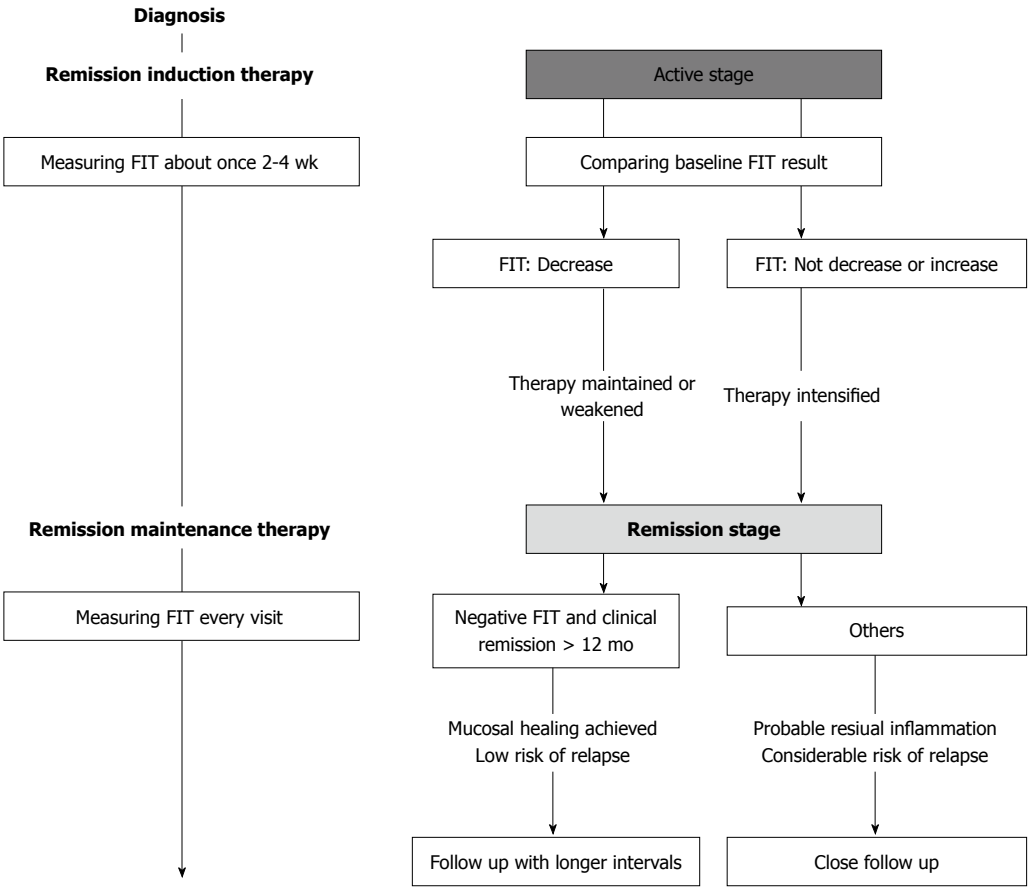

Figure 6 Proposed workflow of the follow-up of Ulcerative colitis patients using fecal immunochemical test. FIT: Fecal immunochemical test.

negative FIT results and that of MES 0 patients as the former are more likely to relapse within one year after colonoscopy/FIT than the latter. In addition, MES 0 patients with a negative FIT at colonoscopy/FIT maintained clinical remission significantly longer than MES 1-3 patients. These results suggest that UC patients who enter clinical remission achieve a negative FIT first, followed by an MES of 0 (complete healing). The relapse rate among patients with a negative FIT who sustained clinical remission for one year or more was the same as the rate among MES 0 patients.

On the basis of our findings, we recommend that UC patients undergo an FIT one year after induction of clinical remission. If the FIT result is negative, then colonoscopy can be safely skipped, and the optimal treatment goal of complete mucosal healing should be considered met. If the FIT result is positive, then physicians should consider performing a colonoscopy or intensifying treatment. Thus, the FIT (an easy, non-invasive and low-cost test) may function advantageously as a substitute for endoscopy to measure mucosal status and risk of relapse in UC patients one year after remission.

There is accumulating evidence that fecal calprotectin, a major protein found in the cytosol of inflammatory cells, is an effective pioneer and is useful for assessing intestinal inflammation[14-16]. Several studies have reported that fecal calprotectin values can predict relapse in UC patients in clinical remission[17-21]. Although these reports indicated that patients with higher fecal calprotectin levels were more likely to relapse within several to 12 mo, no correlation between fecal calprotectin and mucosal status was identified because endoscopic examinations were not included in the studies.

Other reports, which indicate that fecal calprotectin levels can predict endoscopic mucosal healing[22-24], did not investigate risk of relapse. Thus, clear evidence defining the relationship between fecal markers, mucosal status and risk of relapse is lacking. In contrast, our analyses of FIT results as a marker for complete mucosal healing include all 3 variables: Negative FIT results one year or more after UC remission correlated with complete mucosal healing and also with a minimum risk of relapse. Because fecal calprotectin has recently been reported to also correlate with the presence of histological inflammation[25], testing the correlation between negative FIT results and histological remission is one of our future aims.

Reports comparing fecal hemoglobin and calprotectin levels directly as predictors of mucosal status are scarce. Mooiweer *et al*[26] demonstrated that both markers are similarly effective in identifying inflammatory bowel disease (IBD) patients with active endoscopic inflammation. However, to the best

of our knowledge, no reports have compared the predictability of mucosal healing and/or risk of relapse between the two fecal markers. To further understand the roles of these markers in the clinical management of IBD, we aim to conduct such comparative studies in the future.

The FIT has particular advantages over fecal calprotectin testing: Fetal calprotectin is measured using an enzyme-linked immunosorbent assay (ELISA), which is time-consuming and requires specialized techniques, whereas the FIT can be easily measured automatically in a few minutes. In addition, there is significant inter- and intra-assay variability in measures of fecal calprotectin levels using different ELISA diagnostic kits [such as PhiCal Calprotectin ELISA (R-Biopharm, Darmstadt, Germany), Calprest (Eurospital, Trieste, Italy), and Calprotectin ELISA (Bühlmann, Basel, Switzerland)]. The lack of an established assay kit and an optimal cutoff value for detecting mucosal healing/inflammation in UC patients is another major limitation of fecal calprotectin[14-16]. In this regard, the FIT used in this study is the most widely used system worldwide (OC-Sensor neo), and it maintains a consistent standard cutoff (100 ng/mL) for CRC screening that can also be applied as a robust evaluator of mucosal healing in UC patients.

A retrospective design and single-hospital dataset analyses are limitations of this study. However, we argue that the observational nature of the study, which did not require interventions in clinical practice, should limit bias in the results. Despite its limitations, our study revealed that the clinical prognosis of UC patients in remission differs between patients with complete endoscopic remission (MES 0) and those without (MES 1-3). We also demonstrate that in patients who are one year or more removed from UC remission induction, there is a strong positive correlation between negative FIT results, an MES of 0 and better prognosis. We suggest performing the noninvasive FIT in UC patients in prolonged remission (in place of endoscopy) to simplify the assessment of healing and meeting of treatment goals. These findings may greatly improve clinical practice in the evaluation of UC patients, particularly those in clinical remission.

COMMENTS

Background

Mucosal healing is a treatment goal for better prognosis in ulcerative colitis (UC). The authors previously reported that the quantitative fecal immunochemical test (FIT) effectively reflects the mucosal status of UC and that a negative FIT strongly correlates with mucosal healing.

Research frontiers

Currently, the definition of mucosal healing is not yet standardized. Some reports have defined mucosal healing with a Mayo endoscopic subscore (MES) of 0 or 1, whereas others define healing as only an MES of 0. The differences in the clinical outcomes between MES 0 and MES 1 patients have not yet been systematically evaluated. In addition, it has not yet been established whether FIT results can function as a surrogate marker for prognosis in UC patients in clinical remission.

Innovations and breakthroughs

Patients with an MES of 0 were much less likely to relapse than those with an MES of 1. Thus, an MES of 0 should be a treatment goal and an optimal definition of mucosal healing. In addition, this is the first study to demonstrate that FIT results can predict the prognosis of UC. In patients in clinical remission, those with a negative FIT result were less likely to relapse than patients with a positive FIT. Moreover, negative FIT results one year or more after remission induction correlated with an MES of 0 and better prognosis.

Applications

The endoscopic features of the appropriate treatment goal of UC were indicated. The predictability of the FIT as a measure of mucosal status and the prognosis of patients with clinical remission were shown. Patients with a negative FIT demonstrated a lower risk of clinical relapse than those with a positive FIT. Additional results revealed that the risk of relapse in patients in prolonged remission and with a negative FIT were similar to those with an MES of 0. Taken together, these findings would be useful in an economical follow-up of UC patients.

Peer-review

The manuscript by Asuka Nakarai and colleagues describe a retrospective cohort study on ulcerative colitis. This manuscript is prepared with care and detail. Significance of the study is reflected by analysing human data over time. Ethical data and information are provided. Authors declared no conflict of interest.

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ORIGINAL ARTICLE

Retrospective Study

Blood neutrophil-lymphocyte ratio predicts survival after hepatectomy for hepatocellular carcinoma: A propensity score-based analysis

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Abstract

AIM: To investigate whether an elevated preoperative neutrophil-to-lymphocyte ratio (NLR) can predict poor survival in patients with hepatocellular carcinoma (HCC).

METHODS: We retrospectively reviewed 526 patients with HCC who underwent surgery between 2004 and

2011.

RESULTS: Preoperative NLR ⩾ 2.81 was an independent predictor of poor disease-free survival (DFS, $P < 0.001$) and overall survival (OS, $P = 0.044$). Compared with patients who showed a preoperative NLR < 2.81 and postoperative increase, patients who showed preoperative NLR ⩾ 2.81 and postoperative decrease had worse survival (DFS, $P < 0.001$; OS, $P < 0.001$). Among patients with preoperative NLR ⩾ 2.81, survival was significantly higher among those showing a postoperative decrease in NLR than among those showing an increase (DFS, $P < 0.001$; OS, $P < 0.001$). When elevated, alpha-fetoprotein (AFP) provided no prognostic information, and so preoperative NLR ⩾ 2.81 may be a good complementary indicator of poor OS whenever AFP levels are low or high.

CONCLUSION: Preoperative NLR ⩾ 2.81 may be an indicator of poor DFS and OS in patients with HCC undergoing surgery. Preoperative NLR ⩾ 2.81 may be a good complementary indicator of poor OS when elevated AFP levels provide no prognostic information.

Key words: Blood neutrophil-to-lymphocyte ratio; Hepatocellular carcinoma; Liver resection; Prognosis; Postoperative change in neutrophil-to-lymphocyte ratio

Core tip: We retrospectively analyzed a relatively large cohort of patients and used propensity score matching to balance out biases related to patient selection. Our results suggest that preoperative neutrophil-to-lymphocyte ratio (NLR) is a significant predictor of poor overall and disease-free survival. We further suggest that postoperative decrease in NLR is associated with poor survival, although only in patients with high preoperative NLR. Finally, we show that preoperative NLR ⩾ 2.81 may be a good complementary indicator of poor overall survival when elevated alpha-fetoprotein levels provide no prognostic information.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a devastating malignancy that is the third most frequent cause of cancer-associated mortality worldwide. Hepatectomy and transplantation are considered curative treatments for HCC, but long-term survival is far from satisfactory due to the high frequency of tumor recurrence[1,2].

The prognosis of HCC patients who undergo resection varies, as it is dependent on such factors as tumor size, tumor number, vascular invasion, and tumor capsule, but these factors can only be assessed after surgery and so cannot be used for preoperative patient selection. One potential preoperative prognostic indicator is the neutrophil-to-lymphocyte ratio (NLR). This indicator of systemic inflammation is easy and inexpensive to determine[3-7], and elevated pretreatment NLR has been associated with poor outcome in numerous malignancies, including colon cancer[4], gastric cancer[8], HCC[3], and breast cancer[6].

Although studies have suggested that an elevated pretreatment NLR may correlate with a poor outcome in patients with HCC[9-11], other studies failed to detect such a correlation[12-14]. To gain a clearer picture of the influence of preoperative NLR on survival and recurrence after surgery for HCC, we carried out a retrospective study on propensity score-matched patients.

NLR often changes after hepatic resection in HCC patients, perhaps reflecting the shifting balance between inflammatory activity and immune activity. This raises the question of whether a postoperative change in NLR also serves as a predictor of prognosis after surgery. A study of 189 patients with early stage HCC suggested that a postoperative increase in NLR was associated with poorer overall survival and disease-free survival[13], but this has yet to be confirmed in larger samples.

The present study retrospectively analyzed a relatively large sample of Chinese patients with HCC in order to assess the usefulness of both preoperative NLR and postoperative change in NLR as prognostic indicators.

MATERIALS AND METHODS

This research was approved by the Ethics Committee of the Tumor Hospital of Guangxi Medical University. Written informed consent was obtained from participating patients.

Patients

All patients who underwent hepatic resection for primary HCC as initial treatment at the Affiliated Tumor Hospital of Guangxi Medical University between May 2004 and September 2011 were considered for inclusion in the study. Patients were diagnosed with primary HCC when two types of imaging technique showed features typical of HCC, or when one imaging technique gave positive findings and the alpha fetoprotein (AFP) level was > 400 ng/mL. Diagnosis of HCC was confirmed by histopathological examination.

Patients were excluded from the study if they underwent transarterial chemoembolization (TACE),

radiofrequency ablation (RFA), percutaneous ethanol injection, or other anti-tumor therapies before hepatic resection. Patients were also excluded if they suffered from preoperative fever.

Baseline clinical characteristics and laboratory results were recovered from the hospital database.

Definition of NLR

NLR was calculated by dividing the neutrophil count by the lymphocyte count. Preoperative NLR was determined within 7 d of surgery, and postoperative NLR was determined at the first follow-up visit in the outpatient department a month after surgery. Postoperative change in NLR was calculated by dividing postoperative NLR by preoperative NLR. The resulting numerical changes were transformed into a binary outcome of postoperative increase in NLR (when the ratio was $\geqslant 1$) or postoperative decrease in NLR (when the ratio was < 1). For certain analyses, patients were divided into groups with low or high preoperative NLR using a cutoff value of 2.81, as reported in the literature[9,11].

Follow-up visits and outcomes

All patients were followed up 1 mo after liver resection, every subsequent 3 mo during the first postoperative year, and every 6 mo thereafter until 60 mo after surgery or until death. At each follow-up visit, routine blood tests, serum AFP assay, ultrasound and computed tomography (CT), or magnetic resonance imaging (MRI) were performed.

Outcomes were overall survival (OS) and disease-free survival (DFS). DFS was defined as the interval from hepatectomy to imaging-based discovery of tumor relapse. OS was defined as 60 mo for those who survived more than 60 mo and DFS was defined as 60 mo if tumor relapse did not occur within 60 mo.

Propensity score analysis

Since patients were assigned to groups based on a preoperative NLR cut-off rather than randomization, propensity score analysis was used to balance out patient differences related to patient selection for hepatic resection. Propensity scores for all patients were estimated using a logistic regression model, which included all covariates that might have affected patient assignment to a high or low preoperative NLR group, as well as patient survival (Table 1). One-to-one nearest-neighbor matching was performed between high and low preoperative NLR using a 0.1 caliper width[14]. The resulting score-matched pairs were used in subsequent analyses as indicated.

Statistical analysis

Statistical analysis was performed using SPSS 19.0 (IBM, United States). Intergroup differences in categorical data were assessed for significance using χ^2 test, while intergroup differences in continuous data were assessed using the Mann-Whitney *U* test or *t* test. OS and DFS were analyzed using the Kaplan-Meier approach, and differences were assessed for significance using the log-rank test. Independent prognostic factors were identified using the Cox proportional hazards model. $P < 0.05$ served as the threshold of significance.

RESULTS

Study population

Between May 2004 and September 2011, 858 patients underwent hepatectomy for HCC at the Affiliated Tumor Hospital of Guangxi Medical University. Of these, 332 (38.7%) were excluded from our study because they (1) received initial HCC treatment at other centers (n = 288, 33.5%); (2) had already undergone RFA, TACE, percutaneous ethanol injection, or another pre-resection procedure (n = 24, 2.8%); or (3) suffered from preoperative fever (n = 20, 2.3%).

Ultimately, 526 patients (61.5%) were enrolled in the study, of whom 452 (85.9%) received curative hepatectomy. The remaining 74 patients (14.1%) received hepatectomy that was considered palliative because they had macroscopic vessel invasion[15].

Clinicopathological characteristics

Of the 526 patients, 125 (23.8%) had NLR levels higher than the cut-off value and were included in the high NLR group, while the remaining 401 (76.2%) were included in the low NLR group. The two groups were balanced in terms of gender, age, Edmondson grade, surgical margin, Child-Pugh class, and tumor number, as well as levels of albumin, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) (all $P > 0.05$; Table 1). However, the two groups were unbalanced in terms of the presence of hepatitis B surface antigen (HbsAg), liver cirrhosis, tumor capsule, and vascular invasion; levels of AFP, platelets, and total bilirubin; Barcelona Clinic Liver Cancer (BCLC) stage; and tumor size (all $P < 0.05$; Table 1). Propensity score matching was used to generate 111 pairs of patients from the two groups, who showed no significant differences (Table 1).

Survival among all patients and propensity-matched pairs

Among all patients in the study, DFS was 55.4% at 1 year, 37.3% at 3 years, and 19.6% at 5 years. The corresponding OS rates were 78.2%, 57.9%, and 35.6%, respectively. Among propensity-matched pairs of patients, DFS was significantly higher in the low-NLR group than in the high-NLR group at 1, 3, and 5 years (Figure 1A). Similar results were obtained for OS (Figure 1B).

Risk factors for prognosis after hepatectomy

Among all patients in the study, univariate analysis

Table 1 Clinicopathological variables in Chinese patients with hepatocellular carcinoma treated by hepatic resection

Variable	Before propensity matching			After propensity matching		
	NLR < 2.81 *n* = 401	NLR ⩾ 2.81 *n* = 125	*P* value	NLR < 2.81 *n* = 111	NLR ⩾ 2.81 *n* = 111	*P* value
Gender, M/F	358/43	107/18	0.262	94/17	97/14	0.561
Age (yr)	46.8 ± 10.9	47.9 ± 11.5	0.309	46.1 ± 11.6	47.2 ± 11.2	0.487
HbsAg						
Negative	50	25	0.041	18	19	0.857
Positive	351	100		93	92	
Liver cirrhosis						
Yes	338	94	0.023	85	86	0.873
No	63	31		26	25	
AFP (ng/mL)						
< 400	268	70	0.032	67	67	1.000
⩾ 400	133	55		44	44	
Edmonson grade						
Ⅰ-Ⅱ	238	84	0.166	71	71	1.000
Ⅲ-Ⅳ	163	41		40	40	
Surgical margin (cm)						
< 1	195	51	0.126	48	50	0.787
⩾ 1	206	74		63	61	
BCLC stage						
0 or A	180	69	0.044	53	57	0.591
B or C	221	56		58	54	
Child-Pugh class						
A	385	117	0.260	105	105	1.000
B	16	8		6	6	
Tumor number						
Single	293	86	0.363	75	79	0.560
Multiple	108	39		36	32	
Tumor size (cm)	6 (4-8)	8 (5.5-12)	< 0.001	7.5 (6-11)	8 (5-12)	0.769
Tumor capsule						
Complete	181	40	0.010	33	39	0.390
Incomplete	220	85		78	72	
Vascular invasion						
Absent	354	98	0.008	91	91	1.000
Present	47	27		20	20	
Albumin (g/L)	40.1 ± 4.4	39.8 ± 4.1	0.399	40.3 ± 5.1	39.9 ± 4.2	0.583
Platelet count (10^9/L)	180.6 ± 76.6	202.1 ± 84.7	0.008	206.5 ± 82.6	200.91 ± 85.73	0.624
AST (U/L)	41 (36-60)	49 (37-67.5)	0.371	47 (31-70)	49 (37-70)	0.138
ALT (U/L)	40 (29-58)	42 (27.5-54)	0.393	37 (26-59)	42 (33-55)	0.400
Total bilirubin (μmol/L)	12 (9-16.8)	14 (9.9-19.5)	0.010	12.3 (9.2-17.4)	14 (9.9-19.4)	0.051

Data are mean ± SD or median (25^{th}-75^{th} interquartile range) unless otherwise indicated. AFP: Alpha-fetoprotein; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HBsAg: Hepatitis B surface antigen; NLR: Blood neutrophil-to-lymphocyte ratio.

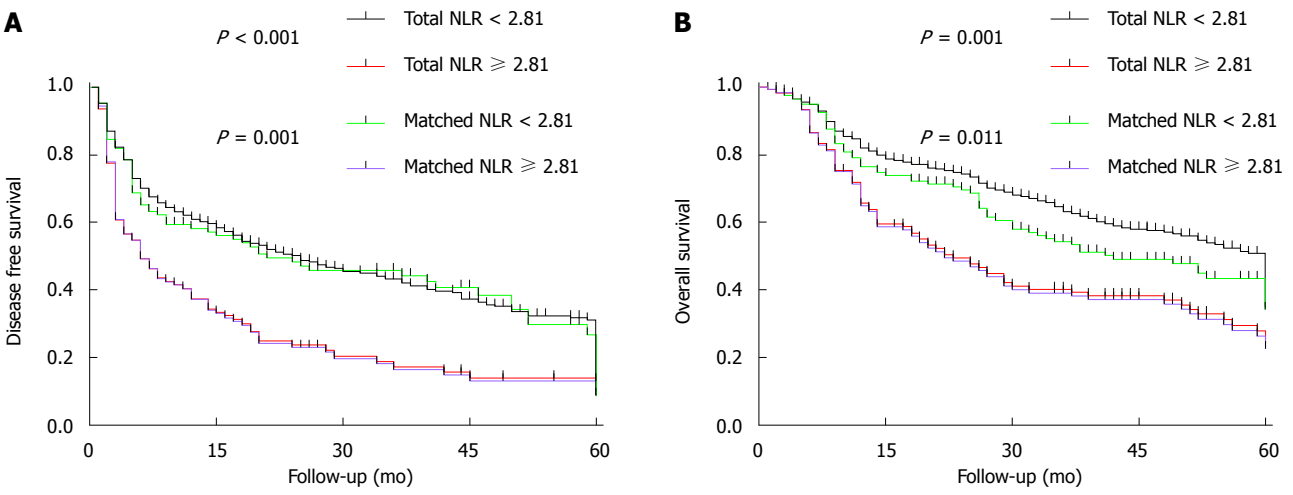

Figure 1 Post-hepatectomy disease-free survival (A) and overall survival (B) of hepatocellular carcinoma patients with high or low neutrophil-to-lymphocyte ratio. Separate curves are shown for the entire cohort (*n* = 526) and the propensity-matched cohort (*n* = 222). NLR: Neutrophil-to-lymphocyte ratio.

Preoperative NLR	Postoperative change in NLR	*n*	Disease-free survival, mo	*P* value
< 2.81	Decrease	227	32.3 ± 1.8	
< 2.81	Increase	124	33.7 ± 2.3	
⩾ 2.81	Decrease	92	20.9 ± 2.4	
⩾ 2.81	Increase	13	2.9 ± 0.5	
< 2.81	Decrease	227	44.6 ± 1.3	
< 2.81	Increase	124	47.7 ± 1.6	
⩾ 2.81	Decrease	92	37.1 ± 2.3	
⩾ 2.81	Increase	13	10.6 ± 2.6	

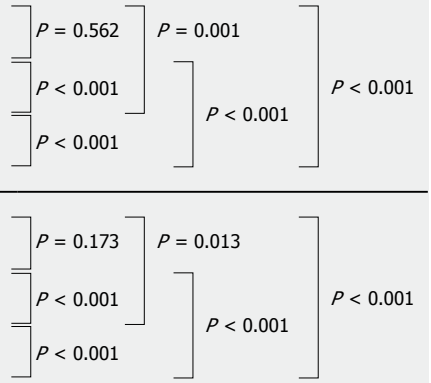

Figure 2 Comparison of survival of Chinese patients with hepatocellular carcinoma, stratified by preoperative neutrophil-to-lymphocyte ratio and postoperative change in neutrophil-to-lymphocyte ratio. NLR: Neutrophil-to-lymphocyte ratio.

Table 2 Multivariate analysis to identify factors predicting poor overall survival and disease-free survival in Chinese patients with hepatocellular carcinoma after hepatectomy

Factor	HR	95%CI	*P* value
Disease-free survival			
AFP > 400 ng/mL	1.493	1.062-2.100	< 0.001
Multiple tumors	1.766	1.385-2.252	< 0.001
Tumor size ⩾ 5 cm	1.313	1.018-1.693	0.036
Vascular invasion	2.656	1.962-3.594	< 0.001
NLR ⩾ 2.81	1.610	1.250-2.075	< 0.001
Overall survival outcome			
Multiple tumors	1.649	1.257-2.16	< 0.001
Tumor size ⩾ 5 cm	1.912	1.407-2.59	< 0.001
Incomplete tumor capsule	1.480	1.139-1.92	0.003
Vascular invasion	2.239	1.496-3.350	< 0.001
NLR ⩾ 2.81	1.333	1.007-1.76	0.044

Calculated over all patients in the study (n = 526). AFP: Alpha-fetoprotein.

identified several factors significantly associated with poor DFS: AFP ⩾ 400 ng/mL, Edmondson grade Ⅲ-Ⅳ, surgical margin < 1 cm, multiple tumors, tumor size ⩾ 5 cm, incomplete tumor capsule, vascular invasion, preoperative NLR ⩾ 2.81, AST ⩾ 80 U/L, and BCLC stage B or C. With the exception of AFP level, all of the aforementioned factors were also found to be significantly associated with poor OS.

Multivariate analysis (Table 2) identified the following independent predictors of poor DFS: AFP ⩾ 400 ng/mL, multiple tumors, tumor size ⩾ 5 cm, vascular invasion, and preoperative NLR ⩾ 2.81. Excluding AFP level, all of these factors were also found to be independent predictors of poor OS.

Postoperative change in NLR as possible prognostic factor

In the complete cohort of 526 patients, postoperative NLR data were available for 456 (86.7%). These fell into the following four subgroups (Figure 2): 227 patients (49.8%) who had a preoperative NLR < 2.81 and showed a postoperative decrease in NLR; 124 (27.2%) who had a preoperative NLR < 2.81 and showed a postoperative increase in NLR; 92 (20.2%) with preoperative NLR ⩾ 2.81 and a postoperative decrease in NLR; and 13 (2.9%) with NLR ⩾ 2.81 and a postoperative increase in NLR.

Compared with the patients who show preoperative NLR < 2.81 and postoperative increase, the patients who show preoperative NLR ⩾ 2.81 and postoperative decrease have worse survival (DFS, P < 0.001; OS, P < 0.001; Figure 2). Among patients with preoperative NLR ⩾ 2.81, survival was significantly higher for those showing a postoperative decrease in NLR than for those showing a postoperative increase (DFS, P < 0.001; OS, P < 0.001; Figure 2).

Prognostic value of preoperative NLR based on AFP levels

Since univariate analysis identified preoperative AFP ⩾ 400 ng/mL as a predictor of poor DFS but not OS, we wanted to examine whether the prognostic value of preoperative NLR varied with AFP level. Analysis of patient subgroups with AFP levels of 200, 400 ng/mL showed that, when elevated, AFP levels provide no prognostic information and that preoperative NLR ⩾ 2.81 may be a complementary indicator of poor OS whenever alpha-fetoprotein (AFP) levels are low or high (Figure 3).

DISCUSSION

The present retrospective study with a relatively large cohort of Chinese HCC patients suggests that elevated preoperative NLR is associated with poor OS and DFS, and that a postoperative increase in NLR is associated with poor survival. This result may not only assist surgeons in predicting HCC patient survival before and after surgery, but also act to remind the surgeon to perform timely adjuvant treatment to improve the

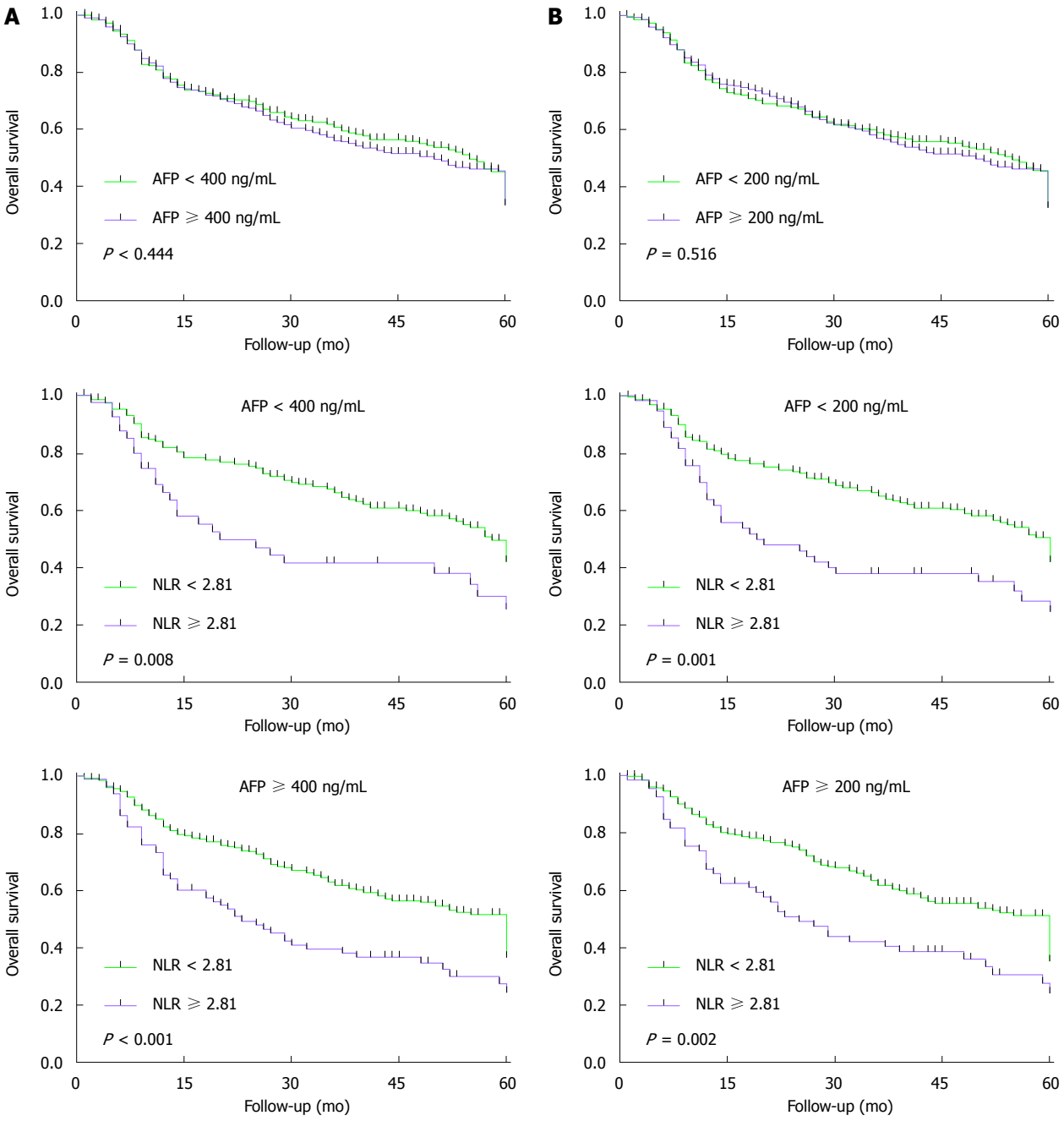

Figure 3 Comparison of overall survival of hepatocellular carcinoma patients stratified based on preoperative alpha-fetoprotein level and on high or low preoperative neutrophil-to-lymphocyte ratio. Patients were grouped using alpha-fetoprotein cut-off values of 400 ng/mL (A), 200 ng/mL (B). Results are shown only for the entire cohort (n = 526). NLR: Neutrophil-to-lymphocyte ratio.

prognosis of patients with preoperative NLR ⩾ 2.81.

In our cohort, elevated serum AFP levels were not significant predictors of poor OS after resection: OS did not vary significantly with preoperative AFP levels of 200, 400 ng/mL. AFP remains controversial as a predictor of HCC patient survival after resection; while some studies have associated elevated serum AFP levels with poor prognosis[11,16-18], others have failed to find such an association[10,13,15,19]. Our results suggest that, when elevated AFP levels provide no prognostic information, preoperative NLR ⩾ 2.81 may be a complementary indicator of poor OS whenever AFP levels are low or high.

Why elevated NLR and postoperative NLR increase should predict poor survival remains unclear, but some studies have proposed explanations. One such explanation is that many patients with elevated NLR have lymphocytopenia, which may contribute to a weak lymphocyte-mediated immune response to tumors[20]. This lymphocyte-mediated response normally aids in the elimination of abnormal cells and in the production of cytokines that inhibit tumor proliferation, invasion, and metastasis[21]. Another possible explanation is that elevated NLR reflects a stronger neutrophil response

and higher numbers of peripheral neutrophils, leading to higher secretion of pro-angiogenic factors such as interleukin-8[22], vascular endothelial growth factor (VEGF)[23,24], and matrix metalloproteinase (MMP)[25,26], which may contribute to tumor growth and therefore to poor prognosis. Studies have indicated that a postoperative NLR increase may reflect that the body has not recovered from tumor control after surgery[27], potentially leading to worse survival.

The findings of the present study should be interpreted with caution in light of several limitations. First, this study is retrospective and based on patients at a single institution. Indeed, more than 85% of our cohort was chronically infected with hepatitis B virus, which is not the case in other parts of the world. Secondly, the cut-off value of NLR was obtained from published papers[9,11]. Thirdly, owing to the distribution of HCC patients, the number of patients who showed preoperative NLR ⩾ 2.81 and postoperative increase was more than 10 times less than others, which may have led to variance in the results.

In conclusion, our results suggest that both preoperative NLR and postoperative change in NLR are predictors of OS and DFS in HCC patients undergoing hepatic surgery. Elevated NLR may be a complementary, or even an alternative, biomarker of survival when elevated AFP levels prove uninformative.

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COMMENTS

Background

An elevated preoperative neutrophil-to-lymphocyte ratio (NLR) may predict poor survival in patients with hepatocellular carcinoma (HCC), but this requires confirmation.

Research frontiers

The prognosis of HCC patients who undergo resection depends on factors such as tumor size and number, vascular invasion, and tumor capsule, but these factors can only be assessed after surgery and so cannot be used for preoperative patient selection. One potential preoperative prognostic indicator is the neutrophil-to-lymphocyte ratio. This indicator of systemic inflammation is easy and inexpensive to determine.

Innovations and breakthroughs

The authors retrospectively analyzed a relatively large cohort of patients and used propensity score matching to balance out biases related to patient selection in order to investigate the impact of preoperative NLR and postoperative NLR on survival. This study will provide more evidence for NLR after curative resection of HCC in the future.

Applications

Preoperative NLR ⩾ 2.81 may be an indicator of poor DFS and OS in patients with HCC undergoing surgery. Preoperative NLR ⩾ 2.81 may be a good complementary indicator of poor OS when elevated AFP levels provide no prognostic information.

Terminology

NLR was calculated by dividing the neutrophil count by the lymphocyte count. Postoperative change in NLR was calculated by dividing postoperative NLR by preoperative NLR. The resulting numerical changes were transformed into a binary outcome of postoperative increase in NLR (when the ratio was ⩾ 1) or postoperative decrease in NLR (when the ratio was < 1).

Peer-review

This is an interesting manuscript that shows the benefit of using an easily available tool for prognosis after resection for HCC.

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ORIGINAL ARTICLE

Observational Study

Serum adipokines might predict liver histology findings in non-alcoholic fatty liver disease

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Author contributions: Jamali R and Aarabi MH proposed the idea and designed the research; Jamali R, Arj A and Razavizade M diagnosed NAFLD and enrolled patients; Jamali R, Arj A, Aarabi MH and Razavizade M collected the data; Jamali R performed the statistical analysis and interpreted the data; Jamali R and Aarabi MH wrote the draft; all authors read and approved the final manuscript.

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Abstract

AIM: To assess significance of serum adipokines to determine the histological severity of non-alcoholic fatty liver disease.

METHODS: Patients with persistent elevation in serum aminotransferase levels and well-defined characteristics of fatty liver at ultrasound were enrolled. Individuals with a history of alcohol consumption, hepatotoxic medication, viral hepatitis or known liver disease were excluded. Liver biopsy was performed to confirm non-alcoholic liver disease (NAFLD). The degrees of liver steatosis, lobular inflammation and fibrosis were determined based on the non-alcoholic fatty liver activity score (NAS) by a single expert pathologist. Patients with a NAS of five or higher were considered to have steatohepatitis. Those with a NAS of two or lower were defined as simple fatty liver. Binary logistic regression was used to determine the independent association of adipokines with histological findings. Receiver operating characteristic (ROC) analysis was employed to determine cut-off values of serum adipokines to discriminate the grades of liver steatosis,

lobular inflammation and fibrosis.

RESULTS: Fifty-four participants aged 37.02 ± 9.82 were enrolled in the study. Higher serum levels of visfatin, IL-8, TNF-α levels were associated independently with steatosis grade of more than 33% [β = 1.08 (95%CI: 1.03-1.14), 1.04 (95%CI: 1.008-1.07), 1.04 (95%CI: 1.004-1.08), $P < 0.05$]. Elevated serum IL-6 and IL-8 levels were associated independently with advanced lobular inflammation [β = 1.4 (95%CI: 1.09-1.8), 1.07 (95%CI: 1.003-1.15), $P < 0.05$]. Similarly, higher TNF-α, resistin, and hepcidin levels were associated independently with advanced fibrosis stage [β = 1.06 (95%CI: 1.002-1.12), 19.86 (95%CI: 2.79-141.19), 560.72 (95%CI: 5.98-5255.33), $P < 0.05$]. Serum IL-8 and TNF-α values were associated independently with the NAS score, considering a NAS score of 5 as the reference value [β = 1.05 (95%CI: 1.01-1.1), 1.13 (95%CI: 1.04-1.22), $P < 0.05$].

CONCLUSION: Certain adipokines may determine the severity of NAFLD histology accurately.

Key words: Non-alcoholic fatty liver disease; Adipokine; Histology; Adiponectin; Visfatin; Resistin; Hepcidin; Interleukin; Tumor necrosis factor

Core tip: Considering the drawbacks of current assays, it seemed reasonable to find appropriate serum biomarkers to define the extent of liver damage in non-alcoholic liver disease (NAFLD). We investigated several key adipokines together with metabolic profiles and liver function tests, providing an advantage over previous studies. We concluded that serum visfatin, IL-8, TNF-α levels were associated with liver steatosis degree; serum IL-6 and IL-8 concentrations correlated with lobular inflammation grade; and TNF-α, resistin, and hepcidin levels correlated with fibrosis stage. The study suggested that certain adipokines might have better accuracy than currently used serum biomarkers to determine NAFLD histology.

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INTRODUCTION

Non-alcoholic liver disease (NAFLD) is a health concern worldwide. The burden of the disease is increasing because of the epidemic of obesity and the development of insulin resistance (IR) syndrome[1]. Liver function tests, metabolic profiles, liver ultrasound and clinical data are used routinely to detect the disease. Considering the limitations of these assays, liver biopsy is still the gold standard method to diagnose NAFLD[2]. However, concerns about the possible complications and invasiveness of the method have limited its application by physicians. It seems reasonable to identify appropriate serum biomarkers to diagnose and define the extent of liver damage in NAFLD. In this regard, interest in the roles of adipokines that are secreted from visceral adipose tissue (VAT) has been increasing.

NAFLD comprises a wide spectrum of liver cell injury that is induced by insulin resistance. Primarily, the accumulation of fat occurs in hepatocytes (simple fatty liver) as a consequence of hepatic insulin resistance. A growing body of evidence supports the view that adipokines modulate these metabolic processes by regulating insulin mediated glucose metabolism, fatty acid utilization and lipid accumulation of visceral tissues. At the later stages of disease, inflammatory phenomena arise that might progress to steatohepatitis and, ultimately, cirrhosis. It has been suggested that the development of steatohepatitis is a consequence of the balance between pro and anti-inflammatory effects of adipokines.

There is a paucity of literature regarding the serum threshold values and efficacy of adipokines in the diagnosis and follow-up of fatty liver patients. In this research, we evaluated certain important adipokines that were reported to be associated with NAFLD, in a cohort of biopsy-proven NAFLD patients[3-9].

The aims of this study were: (1) to evaluate the association of histological findings (steatosis, lobular inflammation and fibrosis) and serum biomarkers (including adipokines, inflammatory cytokines, liver function tests and metabolic profiles); and (2) to determine cut-off values of serum biomarkers to identify the grades of steatosis, lobular inflammation and fibrosis.

MATERIALS AND METHODS

Patient enrolment protocol

This study was conducted in the outpatient gastroenterology clinic of Shahid Beheshti general hospital, from September 2012 to September 2014. Initially, patients with persistent elevated serum aminotransferase levels and well-defined characteristics of a fatty liver *via* abdominal ultrasound (Hitachi EUB 405 apparatus equipped with a convex 3.5 MHz probe) were included (Phase 1)[1,10]. The upper normal limit of the serum aminotransferases level was considered as 40 units per liter[11]. Individuals with a history of alcohol consumption, hepatotoxic medication, viral hepatitis and known liver disease were excluded from the study (Phase 2)[1,12]. Liver biopsy was performed on the remaining patients from phase 2 to confirm diagnosis of NAFLD for final enrolment (Phase 3).

Ethical considerations

The study was conducted according to ethical standards for human experimentation (Helsinki Declaration). The ethics committee of the hospital approved the study protocol (No: 8861). The purpose of study was explained to the participants. They were enrolled in the study upon providing written informed consent.

Sample size calculation

The sample size was n = 54, considering the mean prevalence of NAFLD (P = 28%, α = 0.05, z = 1.96, and d = 0.12), according to a previous study[10].

Laboratory assays

Fasting serum samples were obtained to assess the level of adiponectin, visfatin, resistin, hepcidin, IL-6, IL-8 and tumor necrosis factor (TNF)-α by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions. The following kits were used in this study: Human adiponectin and visfatin ELISA kits (Production numbers: AG-45A-0001 and AG-45A-0006 respectively; ADIPOGEN Inc., South Korea), resistin (human resistin ELISA kit, Biovendor, Czech Republic), hepcidin (Lot: RN- 24429; DEMEDITEC GmbH, Kiel-Wellsee, Germany), IL-6 (Lot: 233737; Bendered Systems GmbH, Vienna, Austria), IL-8 (Lot: ab46032; IL-8 human ELISA kit, Abcam, United States), and TNF-α (Lot: ab46087; TNF-α human ELISA kit, Abcam, United States). Fasting blood glucose, insulin, lipid profiles and liver function tests were performed as previously described[1,10-13].

Liver histology

Percutaneous liver biopsy was performed using a true cut needle (G14). A sample larger than 10 mm or with at least five portal tracts was considered acceptable for histological evaluation. Hematoxylin and Eosin (HE) and Masson's Trichrome stainings were performed to evaluate necroinflammation and fibrosis, respectively. To avoid inter-observer disagreement, a single expert pathologist who was blinded to the patient data interpreted samples. The degree of liver steatosis, lobular inflammation and fibrosis was defined based on the "non-alcoholic fatty liver activity score (NAS)"[14]. Patients with a NAS of five or higher were considered to have NASH. Those with a NAS of two or lower were defined as simple fatty liver[14].

Statistical analysis

Continuous variables were reported as the mean ± SD and categorical variables were shown as counts (percent). The Kolmogorov-Smirnov test was used to assess the distribution of serum adipokines. A χ^2 or *t*-test was applied to assess differences among groups, where appropriate. Binary logistic regression analysis using the standard model was applied to evaluate the association of independent variables (including serum adipokines and clinical data) and liver histology findings.

Hepatic steatosis severity was categorized into four degrees according to the NAS. The first two degrees (0-1) represented no and mild liver steatosis, and the next degrees (2-3) indicated moderate to severe liver steatosis. To define the risk of lower liver steatosis versus a more advanced degrees of steatosis, we considered the patients with steatosis grades of less than 33% as the "mild group". Meanwhile, those with higher degrees (2-3) were merged to form the "moderate to severe group".

The lobular inflammation range was graded from 0 to 3 by the NAS. To estimate the risk of lower lobular inflammation against more advanced grades, we labeled the individuals with lobular inflammation of less than two foci per HPF (grade 1) as the "mild group". At the same time, those with higher lobular inflammation grades (2-3) were combined to form the "moderate to severe group".

Hepatic fibrosis content was categorized into 5 stages based on NAS. The former two stages (0-1) demonstrated none/mild fibrosis and the latter stages stand for more advanced fibrosis (2-4). In order to determine the probability of lower fibrosis versus more advanced fibrosis, we labeled the subjects with perisinusoidal or periportal (stage 1) as the "mild group". Those with higher fibrosis stages (2-4) were mixed to form the "moderate to severe group".

For the regression model, liver steatosis, lobular inflammation, fibrosis stage, and NAS were employed as dependent variables; Steatosis grade of less than 33%, lobular inflammation of less than two foci per high powered field (HPF), fibrosis stage of one (perisinusoidal or periportal), and a NAS of five or higher were set as the reference groups, respectively. Standardized correlation coefficient (OR) with the 95%CI was calculated. Serum adipokines that were independently associated with the histological findings were selected for receiver operating characteristic (ROC) analysis. ROC analysis explored the serum adipokines' cut-off values and their sensitivities and specificities to discriminate higher grades of liver steatosis, lobular inflammation and fibrosis. Values with the highest sum of the sensitivity and specificity were reported as the best cut-off values. All statistical analyses were performed by SPSS, version 17 (SPSS, Chicago, United States). The probability of a difference between groups was considered statistically significant if the two-sided P value was less than 0.05.

RESULTS

Seventy participants presumed to have NAFLD were evaluated from September 2012 to September 2014 (phase 1). Reasons for leaving certain patients out of the study were patient refusal to participate in the study (n = 8), normalization of ALT during the lead-in phase (n = 6), autoimmune hepatitis (n = 1) and viral

Table 1 Clinico-demographic and laboratory data of the participants

Variable	Total	Simple fatty liver	Non-alcoholic steatohepatitis
	n = 54	*n* = 2	*n* = 28
Age (yr)	37.02 ± 9.82	27.00 ± 2.82	35.00 ± 8.47
Male gender, *n* (%)	35 (64.8)	2 (100)	17 (60.7)
Waist circumference (cm)	102.13 ± 2.69	101.00 ± 42.24	101.57 ± 2.71
Body mass index (kg/m^2)	30.55 ± 3.97	28.09 ± 7.77	29.92 ± 3.79
Diabetes mellitus present, *n* (%)	12 (22.2)	0 (100)	11 (39.3)
Metabolic syndrome present, *n* (%)	36 (66.7)	1 (50)	21 (75)
Adiponectin (mg/L)	8.14 ± 2.91	8.20 ± 2.67	7.00 ± 0.28
Visfatin (ng/mL)	19.96 ± 17.5	5.40 ± 0.84	18.34 ± 16.18
Resistin (mg/mL)	2.51 ± 1.08	1.70 ± 1.83	2.10 ± 1.04
Hepcidin (ng/mL)	64.0 ± 0.62	48.50 ± 0.38	75.00 ± 0.49
Tumor necrosis factor-alpha (pg/mL)	2.68 ± 19.32	0.96 ± 9.05	3.68 ± 20.93
Interleukin 6 (pg/mL)	7.59 ± 5.75	4.70 ± 0.24	7.41 ± 4.78
Interleukin 8 (pg/mL)	27.41 ± 24.99	13.60 ± 13.01	38.60 ± 28.21
Alanine aminotransferase (U/L)	65.91 ± 36.11	37.00 ± 0.00	82.10 ± 39.25
Aspartate aminotransferase (U/L)	42.18 ± 20.48	25.00 ± 4.24	49.67 ± 24.14
Alkaline phosphatase (U/L)	181.50 ± 76.14	144.21 ± 42.41	180.23 ± 45.22
Gamma glutamyl transpeptidase (U/L)	54.12 ± 62.55	44.40 ± 30.54	55.40 ± 33.26
Fasting blood sugar (mg/dL)	98.41 ± 14.12	98.25 ± 16.31	103.0 ± 0.00
Insulin (mU/L)	15.16 ± 13.42	10.68 ± 3.65	17.40 ± 18.00
Triglyceride (mg/dL)	150.09 ± 70.18	60.20 ± 9.70	167.57 ± 77.19
Total cholesterol (mg/dL)	177.55 ± 34.17	165.85 ± 40.65	183.77 ± 37.76
Low density lipoprotein cholesterol (mg/dL)	100.89 ± 29.03	98.75 ± 35.42	103.15 ± 32.19
High density lipoprotein cholesterol (mg/dL)	48.51 ± 9.06	55.05 ± 7.14	48.66 ± 9.67

Data are presented as mean ± SD unless otherwise noted. Patients with non-alcoholic activity score (NAS) of five or higher were considered to have non-alcoholic steatohepatitis. Those with NAS equal to two or lower were defined as simple fatty liver[14]. NAFLD: Non-alcoholic fatty liver disease.

hepatitis (*n* = 1) (phase 2). Finally, fifty-four patients with biopsy proven NAFLD were included in the study (phase 3). The clinico-demographic and laboratory data of the participants are presented in Table 1.

Participants showed NAS of 4.87 ± 1.71. The frequency of histological findings in the study population is depicted in Figure 1.

Binary logistic regression showed a positive association between serum visfatin, IL-8 and TNF-α level and the grades of steatosis. Similarly, serum IL-6 and IL-8 levels were independently associated with the degrees of lobular inflammation. Serum TNF-α, resistin and hepcidin levels were independently associated with perisinusoidal fibrosis stage. Serum IL-8 and TNF-α values were positively associated with NAS (Table 2).

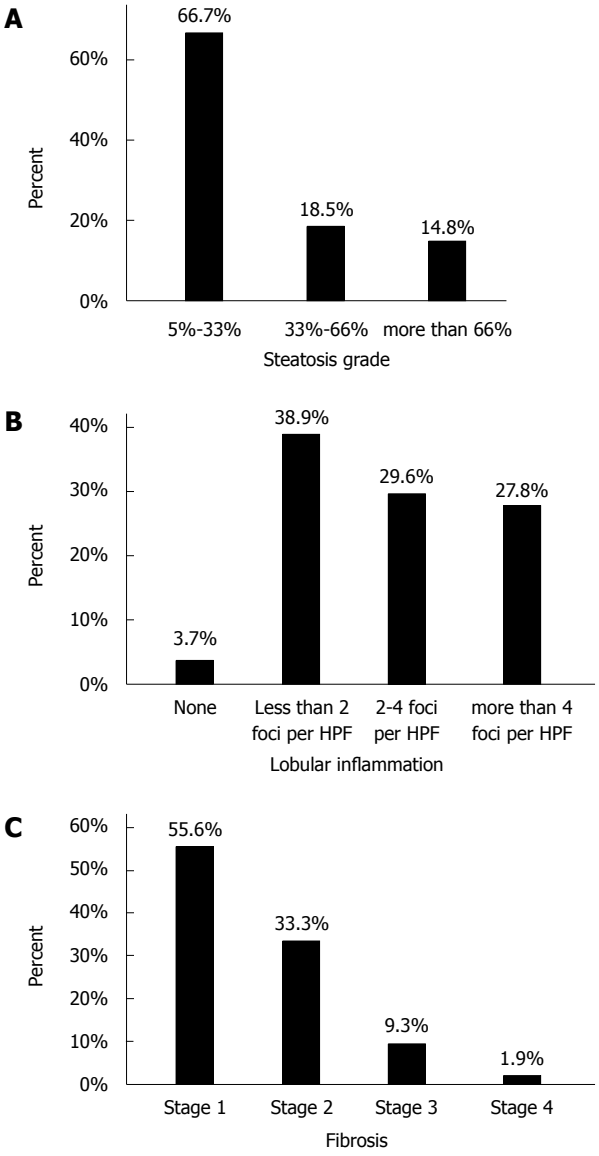

Figure 1 Frequency of histological findings in the participants. Frequency of patients with different degrees of steatosis (A), lobular inflammation grade based on foci of lobular inflammation in high power field of microscopic view (B), and fibrosis stage (C) are presented.

The ROC curves with calculated AUC (± 95%CI) to determine the best cut-off values of serum adipokines to differentiate histological groups are shown in Figure 2. The sensitivities and specificities of the cut-off values of biomarkers to identify histological groups appear in Table 3.

DISCUSSION

This study concluded that serum visfatin, IL-8 and TNF-α levels were independently associated with liver steatosis degree; serum IL-6 and IL-8 concentrations were independently associated with lobular inflammation grade; and TNF-α, resistin and hepcidin levels were independently associated with fibrosis stage in a cohort of biopsy-proven NAFLD patients. Moreover, the best cut-off values for the above-mentioned

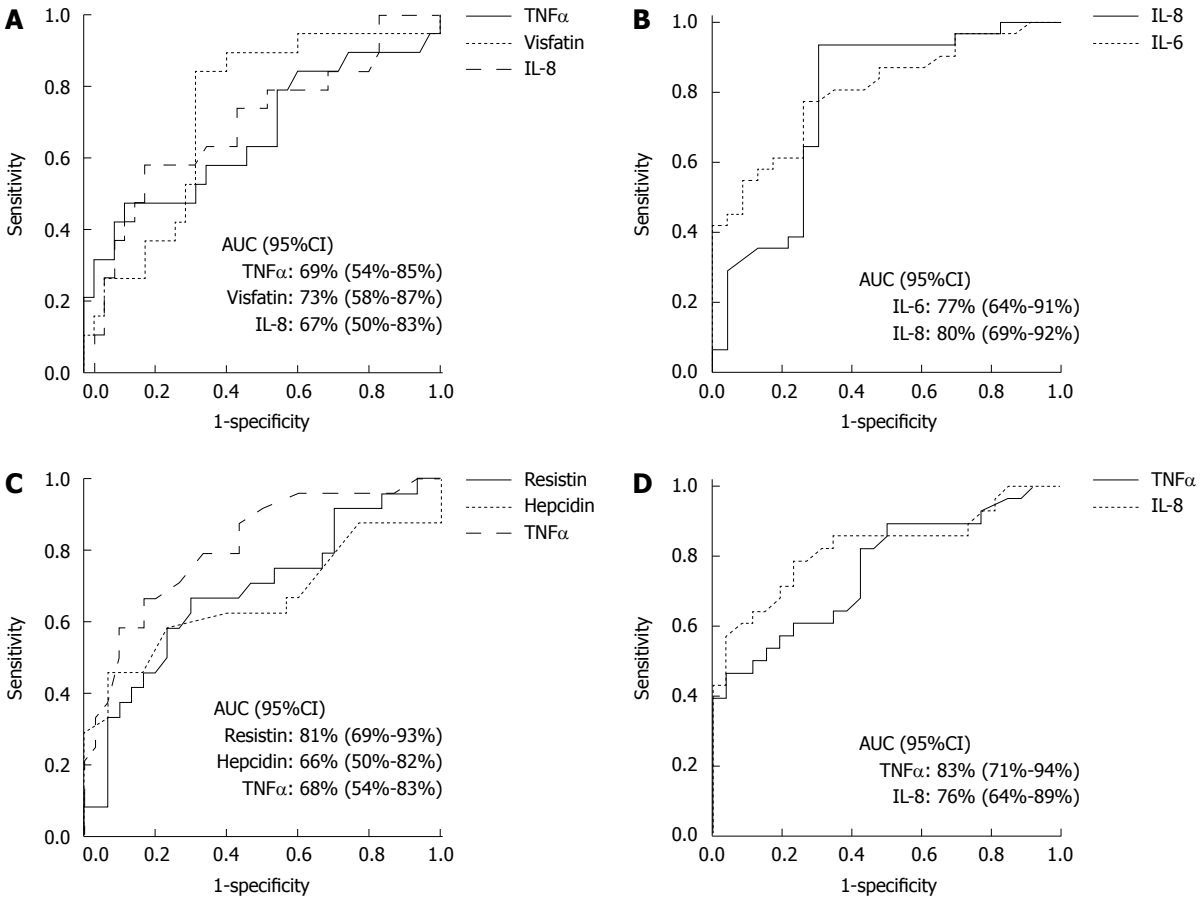

Figure 2 Receiver operating characteristic analysis to determine cut-off values of serum adipokines for differentiating histological severity. A: ROC curve of serum TNF-α, Visfatin, and IL-8 levels to differentiate steatosis degree of less than 33% from more advanced degrees of steatosis; B: ROC curve of serum IL-6 and IL-8 levels to differentiate lobular inflammation grade of less than two foci per high power field from more advanced grades of inflammation; C: ROC curve of serum Resistin, Hepcidin, and TNF-α levels to differentiate fibrosis stage of perisinusoidal or periportal from more advanced stages of fibrosis; D: ROC curve of serum TNF-α and IL-8 levels to differentiate steatohepatitis from simple fatty liver based on non-alcoholic fatty liver disease activity score. AUC: Area under curve; IL: Interleukin.

Table 2 Association between histological findings and serum adipokine levels

Adipokine	OR	95%CI	*P* value
Steatosis degree			
Visfatin	1.08	1.030-1.14	0.001
TNF-α	1.04	1.004-1.08	0.030
Interleukin 8	1.04	1.006-1.07	0.020
Lobular inflammation grade			
Interleukin 6	1.4	1.090-1.80	0.008
Interleukin 8	1.07	1.003-1.15	0.040
Fibrosis stage			
Resistin	19.86	2.790-141.19	0.003
Hepcidin	560.72	5.980-5255.33	0.006
TNF-α	1.06	1.002-1.12	0.040
NAS			
Interleukin 8	1.05	1.01-1.10	0.040
TNF-α	1.13	1.04-1.22	0.004

TNF-α: Tumor necrosis factor-alpha; NAS: Non-alcoholic fatty liver activity score.

serum adipokines were calculated to identify the liver histological findings.

The associations between certain adipokines with NAFLD were evaluated in previous reports[3-9]. We investigated several key adipokines, together with metabolic profiles and LFT, which provided an advantage over the previous studies. To improve the accuracy of the study, the cases were recruited from a cohort of biopsy-proven NAFLD patients. We used NAS for to determine the severity of the liver histology. Notably, NAS is a valid scoring system for NAFLD that differentiates the spectrum of disease with an acceptable reliability and validity[14].

Visfatin is a new adipokine with proinflammatory and metabolic properties. It is increased in IR syndrome. The expression of visfatin in VAT facilitates the maturation of preadipocyte cells to differentiated adipocytes (Paracrine effect)[3]. This fact might explain the correlation between serum visfatin levels and hepatic steatosis degree in our study. Visfatin is also associated with body fat mass in alcoholic fatty liver disease[15]. Previous studies have reported a correlation between visfatin and fibrosis stage, but not with steatosis or lobular inflammation grade in NAFLD[16]. Meanwhile, an increase in serum visfatin was shown to be associated with portal inflammation[17].

TNF-α is a pro-inflammatory cytokine and is asso-

Table 3 Best cut-off values of serum adipokine levels to differentiate histological groups according to receiver operating characteristic analysis

Adipokine	Serum concentration	Sensitivity (%)	Specificity (%)
TNF-α (pg/mL)	2.13	74	58
Visfatin (ng/mL)	13.00	84	69
Interleukin 8 (pg/mL)	24.25	58	66
Cut-off values of serum adipokine levels to differentiate lobular inflammation grade of less than 2 foci per high power field from more advanced grades of inflammation.			
Interleukin 6 (pg/mL)	3.70	94	70
Interleukin 8 (pg/mL)	13.00	77	74
Cut-off values of serum adipokine levels to differentiate perisinusoidal or periportal fibrosis from more advanced stages of fibrosis.			
Resistin (mg/mL)	1.65	79	66
Hepcidin (ng/mL)	45.00	63	60
TNF-α (pg/mL)	2.44	67	70
Cut-off values of serum adipokine levels to differentiate steatohepatitis from simple fatty liver group based on non-alcoholic fatty liver disease activity score (NAS).			
Interleukin 8 (pg/mL)	9.80	82	54
TNF-α (pg/mL)	2.44	71	81
Cut-off values of serum adipokine levels to differentiate steatosis degree of less than 33% from more advanced degrees of steatosis.			

TNF-α: Tumor necrosis factor-alpha.

ciated with hepatic IR in NAFLD[4]. It mediates the early stage of NAFLD by fat accumulation in hepatocytes. In addition, it facilitates disease progression to a more advanced stage[18]. The relationship between serum TNF-α and liver steatosis and fibrosis in our research is in line with previous observations.

IL-8 is also a pro-inflammatory cytokine that activate monocytes and attracts polymorphonuclear leukocytes to the site of inflammation[19]. It is increased in obese individuals with IR. In accordance with the literature, our results showed that serum IL-8 was associated with steatosis degree and lobular inflammation[5].

IL-6 is a liver and adipose tissue-derived proinflammatory cytokine that is implicated in hepatic and skeletal muscle IR. IL-6 is thought to act as a second hit in the pathophysiology of NAFLD, causing the progression of simple fatty liver to NASH[19]. The correlation of IL-6 with lobular inflammation grade in our study is comparable to the findings by other groups[6,20,21].

With regard to hepcidin, the circulatory level was strongly associated with fibrosis stage in our study. Nevertheless, a previous found no correlation between hepcidin and histological findings[7]. Body iron stores in NAFLD regulate hepcidin levels[22]. Therefore, it seems reasonable to adjust for patients iron storage when evaluating hepcidin levels in NAFLD patients.

Resistin is an adipokine that is considered an indicator of IR in obesity[23]. However, the pathophysiological role of resistin in NAFLD is not clear. In this study, we observed that serum resistin levels were related with fibrosis stage. On the other hand, advanced liver fibrosis was associated with reduced resistin concentration in chronic hepatitis C patients with normal body weight, glucose and lipid profiles[24]. Previous studies demonstrated a correlation between high serum resistin levels and the presence of steatosis and necroinflammation in NAFLD[8,25]. Meanwhile, another study demonstrated an association of low serum resistin levels with excessive fat accumulation in the liver[26].

Adiponectin is a well-known adipokine that regulates hepatic IR[27]. It was suggested that adiponectin might be related to steatosis grade and the severity of NAFLD; however, its definitive role remains to be addressed[28]. A decrease in serum adiponectin is the primary event in children with NAFLD before the rise of inflammatory cytokines and the development of overt diabetes[9,29]. One previous study showed that adiponectin could predict patients with higher necroinflammatory grade and fibrosis stage from those with milder histological findings[30]. Another study showed that adiponectin is related to hepatic fat content and not to necroinflammatory activity and fibrosis stage[31]. Meanwhile, our study showed no correlation between adiponectin and liver histology. This study, despite its advantages, suffers from several drawbacks: first, the study was performed in a single institution; therefore, the findings need to be generalized with caution. Second, our study was cross-sectional, which limited the interpretation of causal associations.

There is currently no defined "normal range" for serum adipokines. Moreover, adipokine levels might fluctuate over time according to the metabolic environment. These concerns might explain the differences in the results of the above-mentioned studies with our results. Further well-controlled prospective studies to determine the association of VAT-derived proteins (including proinflammatory cytokines and polypeptide hormones) and liver histological findings are recommended.

The associations of some important adipokines, together with the currently used serum biomarkers, with the liver histological findings were evaluated. Certain adipokines were independently associated with the liver histological findings. Finally, the best cut-off values of these serum adipokines were determined to detect the severity of liver steatosis, lobular inflammation and fibrosis.

In conclusion, this study suggested that certain adipokines might determine accurately the severity of NAFLD based on histological findings.

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COMMENTS

Background

Non-alcoholic liver disease (NAFLD) is a health concern worldwide. The burden of disease is increasing because of an epidemic of obesity. Considering the limitations of current modalities, finding an appropriate serum biomarker to diagnose and assess the severity of liver damage in NAFLD is crucial.

Research frontiers

The roles of adipokines in the pathogenesis of NAFLD have received research interest recently. Nevertheless, there is a paucity of studies that used serum levels of adipokines in the diagnosis and follow up of NAFLD patients.

Innovations and breakthroughs

The associations between certain adipokines with NAFLD were evaluated in previous reports. The authors investigated several key adipokines together with metabolic profiles and LFT, providing an advantage over the previous studies. To improve the accuracy of the study, the cases were selected from a cohort of biopsy-proven NAFLD patients. To assess the severity of NAFLD based on histology, we applied NAS, a valid scoring system for NAFLD that categorizes the spectrum of disease with acceptable reliability and validity.

Applications

This study suggested that certain adipokines might determine accurately the presence and severity of NAFLD.

Peer-review

This manuscript evaluated the association between histological grade of NAFLD and serum biomarkers, and suggested cut-off values of the biomarkers for NASH. The authors used a direct method to define NASH, and measured various biomarkers related to adiposity and inflammation. Finally, the authors showed successfully that the indices were related to each component of NASH.

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ORIGINAL ARTICLE

Prospective Study

Usefulness of portal vein pressure for predicting the effects of tolvaptan in cirrhotic patients

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Author contributions: Nakagawa A, Atsukawa M, Kondo C, Okubo T, Arai T and Itokawa N designed the study and measured porta vein pressure; Atsukawa M and Tsubota A contributed equally to the preparation of this manuscript; Tsubota A, Narahara Y and Iwakiri K supervised the study; all authors have read and approved the final version to be published.

Institutional review board statement: The protocol was approved by the Ethics Review Board of Nippon Medical School Chiba Hokusoh Hospital (approval No. 526012). All patients and their families received a sufficient explanation of the aim and contents of this study before the entry.

Conflict-of-interest statement: The authors declare that they have no conflict of interest.

Informed consent statement: All patients and their families received a sufficient explanation of the aim and contents of this study before the entry. Patients who provided written informed consent participated in this study. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation and with Helsinki Declaration of 1975, as revised in 2008.

Data sharing statement: No additional data are available.

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Abstract

AIM: To elucidate influencing factors of treatment response, then tolvaptan has been approved in Japan for liquid retention.

METHODS: We herein conducted this study to clarify the influencing factors in 40 patients with decompensated liver cirrhosis complicated by liquid retention. Tolvaptan was administered at a dosage of 7.5 mg once a day for patients with conventional diuretic-resistant hepatic edema for 7 d. At the initiation of tolvaptan, the estimated hepatic venous pressure gradient (HVPG) value which was estimated portal vein pressure was measured using hepatic venous catheterization. We analyzed the effects of tolvaptan and influencing factors associated with treatment response.

RESULTS: Subjects comprised patients with a median age of 65 (range, 40-82) years. According to the Child-Pugh classification, class A was 3 patients, class B was 19, and class C was 18. Changes from the baseline in body weight were -1.0 kg (P = 2.04 × 10^{-6}) and -1.3 kg (P = 1.83 × 10^{-5}), respectively. The median HVPG value was 240 (range, 105-580) mmH_2O. HVPG was only significant influencing factor of the weight loss effect. When patients with body weight loss of 2 kg or greater from the baseline was defined as responders, receiver operating characteristic curve analysis showed that the optimal HVPG cutoff value was 190 mmH_2O in predicting treatment response. The response rate was 87.5% (7/8) in patients with HVPG of 190 mmH_2O or less, whereas it was only 12.5% (2/16) in those with HVPG of greater than 190 mmH_2O (P = 7.46 × 10^{-4}). We compared each characteristics factors between responders and non-responders. As a result, HVPG (P = 0.045) and serum hyaluronic acid (P = 0.017) were detected as useful factors.

CONCLUSION: The present study suggests that tolvaptan in the treatment of liquid retention could be more effective for patients with lower portal vein pressure.

Key words: Tolvaptan; V_2 receptor antagonist; Portal vein pressure; Hepatic venous pressure gradient; Decompensated chirrosis

Core tip: To clarify the factors influencing the effect of tolvaptan, a V_2 receptor antagonist, in patients with decompensated liver cirrhosis complicated by liquid retention, we conducted this study. As a result, hepatic venous pressure gradient (HVPG) was the only significant factor that influenced the weight loss effect of tolvaptan. The response rate was 87.5% (7/8) in patients with HVPG of 190 mmH_2O or less, whereas it was only 12.5% (2/16) in those with HVPG of greater than 190 mmH_2O. The present study suggests that tolvaptan in the treatment of liquid retention related to decompensated liver cirrhosis could be more effective for patients with lower portal vein pressure.

Nakagawa A, Atsukawa M, Tsubota A, Kondo C, Okubo T, Arai T, Itokawa N, Narahara Y, Iwakiri K. Usefulness of portal vein pressure for predicting the effects of tolvaptan in cirrhotic patients. *World J Gastroenterol* 2016; 22(21): 5104-5113 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i21/5104.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i21.5104

INTRODUCTION

Liquid retention is a primary complication associated with decompensated liver cirrhosis. Ascites develops at an incidence of approximately 50% within 10 years of the onset of liver cirrhosis[1]. Existence of ascites reduces dietary intakes and deteriorates nutritional statuses, which, in turn, have a negative impact on the quality of life of liver cirrhosis patients[2,3]. Furthermore, the 5-year survival rate after the development of ascites is reportedly 45%[1].

Resting, salt restriction, and therapy with diuretics, such as loop diuretics and anti-aldosterone drugs, have been performed as conventional treatments for ascites related to liver cirrhosis[4,5]. Loop diuretics reduce the reabsorption of sodium and potassium by inhibiting sodium/potassium/chloride cotransporters in the ascending limb of Henle's loop. Anti-aldosterone drugs promote sodium excretion and consequently decrease the excretion of potassium by inhibiting aldosterone receptors. However, the effects of these diuretics are compromised by the progression of liver cirrhosis, leading to electrolyte abnormalities, including hyposodiumemia, a reduction in plasma osmotic pressure, and kidney hypofunction due to a decrease in renal blood flow. The effects of the loop diuretic, furosemide, were previously suggested to be attenuated in patients with liver cirrhosis characterized by a decrease in serum albumin level and reduction in renal blood flow/the glomerular filtration rate[6,7]. If ascites is not improved by these treatments, it is defined as refractory ascites, which is treated with abdominal paracentesis, albumin reinfusion, peritoneal venous shunt (Denver shunt), cell-free and concentrated ascites reinfusion therapy (CART), and transjugular intrahepatic portosystemic shunt (TIPS), but not liver transplantation[4,6,7]. However, there are quite a few patients who are not able to receive these treatments due to complications or conditions that do not meet the indication criteria.

On the other hand, previous studies reported that the V_2 receptor antagonist, tolvaptan, exhibited diuretic effects on heart failure and hyposodiumemia[8-11]. The antidiuretic hormone, vasopressin, enhances water permeability and promotes water reabsorption through V_2 receptors, which exist in the renal collecting ducts. Tolvaptan has been shown to inhibit the vasopressin-related reabsorption of water, thereby increasing water excretion without enhancing the excretion of electrolytes (water-diuretic actions). Since tolvaptan acts on the vascular side around the renal collecting ducts, it differs from the loop diuretic, furosemide. Therefore, its actions are not influenced by a kidney hypofunction-related decrease in the glomerular filtration rate or hypoalbuminemia[12]. Previous studies indicated that tolvaptan prevented conventional diuretic-induced hyposodiumemia in patients with liquid retention[10,13]. Sakaida *et al*[14] conducted a clinical study of tolvaptan for cirrhotic patients with liquid retention and reported increases in the initial 24-h urine volume, even in those with low serum albumin levels. Zhang *et al*[15] indicated that adverse reactions to tolvaptan administration with a daily

dosage of 15 mg included thirst and dry mouth, which were tolerable and safe. Accordingly, tolvaptan for liquid retention in cirrhotic patients who do not respond to conventional diuretics, such as loop diuretics, has been approved in Japan in 2013.

However, not all patients with liquid retention respond to tolvaptan. Furthermore, little is known about the characteristics of patients who respond well to tolvaptan and factors predictive of the therapeutic effect. The present study was conducted to clarify the baseline factors that influence the effect of tolvaptan in cirrhotic patients with conventional diuretic-resistant liquid retention.

MATERIALS AND METHODS

Study design

Forty-seven patients with decompensated liver cirrhosis and liquid retention (pleural effusion, ascites, or lower-limb edema) were recruited for this prospective study in Nippon Medical School Chiba Hokusoh Hospital between September 2013 and August 2015. Patients were eligible for enrollment if they fulfilled the following criteria: (1) patients aged 20 to 85 years; (2) patients diagnosed as liver cirrhosis based on the results of imaging modality (abdominal CT or ultrasonography) or proven by liver biopsy; (3) conventional diuretic-resistant patients in whom liquid retention was not improved with furosemide at a dosage of 20 mg/d or more and/or spironolactone at a dosage of 25 mg/d or more for at least 7 d with salt-restricted diet (5-7 g salinity/day) in-hospital or on an outpatient basis; and (4) patients in whom body weight before breakfast was stable (within the range of ± 1 kg) during the pretreatment observation period. Criteria for exclusion included: (1) uncontrollable hepatocellular carcinoma, such as the Barcelona clinic liver cancer (BCLC) stage D. BCLC stage D is end-stage hepatocellular carcinoma in a patient with disturbed liver function (Child-Pugh C) and/or performance status 3-4, and with an average predicted survival of 3 months; (2) esophageal varices with requiring treatment; (3) existence of portal vein thrombosis based on imaging modality (abdominal CT or ultrasonography); (4) hepatic encephalopathy stage 2 or higher according to The West Haven classification of hepatic encephalopathy including Asterixis[16,17]; (5) type 1 hepatorenal syndrome; and (6) a serum sodium level of 147 mEq/L or higher. All patients and their families received a sufficient explanation of the aim and contents of this study before the entry. Patients who provided written informed consent participated in this study. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation and with Helsinki Declaration of 1975, as revised in 2008. The protocol was approved by the Ethics Review Board of Nippon Medical School Chiba Hokusoh Hospital (approval No. 526012). All patients and their families received a sufficient explanation of the aim and contents of this study before the entry.

Treatment protocol

Patients were initially instructed to receive salt-restricted diet therapy (5 to 7 g/d) and conventional diuretics for at least 7 d. Tolvaptan (SAMUSKA, Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) was orally administered at a dosage of 7.5 mg once a day. Water intake was not restricted during the administration of tolvaptan. No albumin preparation was infused, and ascites and pleural effusion were not removed by paracentesis during the first 7 d of tolvaptan treatment. Based on previous clinical studies using tolvaptan, patients with a decrease of 2 kg or greater from the baseline in body weight were regarded as responders[18,19].

Laboratory tests

Body weight and 24-h urine volume were daily measured before the administration of tolvaptan and during at least 7 d of treatment. Body weight was measured at the time of awaking. Clinical symptoms and vital signs (blood pressure, pulse rate, body temperature, and arterial blood oxygen saturation) were closely monitored every day. Biochemical tests (serum sodium, creatinine, urea nitrogen, albumin, and blood ammonia levels) and urinalysis (urinary osmotic pressure) were performed at 1, 3, 5 and 7 d of treatment.

Measurement of portal vein pressure

At the initiation of tolvaptan, the estimated portal vein pressure [*i.e.*, the hepatic venous pressure gradient (HVPG)][20,21] was measured using hepatic venous catheterization to investigate whether or not HVPG influenced the response of tolvaptan, when patients agreed with the optional HVPG measurement study. The right internal jugular vein (or the left internal jugular vein when the right-sided puncture was difficult or failed) was punctured with an 18-gauge needle, and subsequently a 5F sheath (Super Sheath: MEDKIT, Tokyo) was inserted along a guide wire. A 2.9F balloon catheter (Selecon MP catheter 2: TERUMO CLINICAL SUPPLY, Gifu, Japan) was then inserted into the inferior vena cava (IVC) to measure IVC pressure, which was used as a zero adjustment in portal vein pressure measurement. The balloon catheter was further inserted into the right hepatic vein to occlude it with the balloon. Hepatic venography was performed using Iopamidol (Bayer Medicine, Osaka, Japan) to confirm retrograde contrast enhancement involving the portal trunk and the presence of a hepatic vein-hepatic vein shunt and portal thrombosis. Wedged hepatic venous pressure (WHVP) was subsequently measured, and the balloon was removed to determine the free hepatic venous pressure (FHVP). The difference between WHVP and FHVP, which is equal to HVPG, was

Table 1 Demographic and clinical characteristics at baseline

Characteristics	*n* = 40
Age (yr)	65 (40-82)
Gender (M/F)	26/14
Body weight (kg)	61.9 (44.8-88.5)
Liver disease etiology	
Hepatitis B/Hepatitis C/Alcohol/PBC/PSC/NASH	3/15/15/3/1/3
Child-Pugh classification A/B/C	3/19/18
Total bilirubin (mg/dL)	1.0 (0.4-26.2)
Serum albumin (g/dL)	2.6 (1.6-3.7)
Serum creatinine (mg/dL)	0.95 (0.45-6.45)
Serum eGFR (mL/min/1.73 m^2)	60 (8-112)
Serum sodium (mEq/L)	139 (124-146)
Serum hyaluronic acid (ng/mL)	420.7 (122-6984)
BUN (mg/dL)	19 (8.1-81.8)
Urine osmolality (mOsm/L)	414.5 (254-954)
Hepatic venous pressure gradient (mmH_2O)[1]	240 (105-580)
Dose of furosemide (mg/d)	37.0 ± 29.5
Dose of spironolactone (mg/d)	43.4 ± 26.8
Hepatocellular carcinoma (with/without)	12/28
Esophageal varix (with/without)	25/15

[1]Hepatic venous pressure gradient was measured in 24 patients. Categorical variables are given as number. Almost continuous variables are given as median (range). Dose of furosemide and spironolactone are given as mean ± SD). BUN: Blood urea nitrogen; PBC: Pimary biliary cirrhosis; PSC: Primary sclerosing cholangitis; NASH: Nonalcoholic steatohepatitis; eGFR: Estimated glomerular filtration rat.

regarded as the estimated portal vein pressure. Seven days after the start of tolvaptan, HVPG was repeatedly measured using the same procedures to evaluate the influence of tolvaptan on portal vein pressure, when patients agreed with the optional HVPG measurement study.

Statistical analysis

Changes in 24-h urine volumes and body weights after the administration of tolvaptan were evaluated using Wilcoxon's signed rank test. Subjects were divided into two groups based on the medians of baseline values in quantitative variables, and the two groups were compared using the Mann-Whitney *U*-test. Categorical data were analyzed using the Fisher's exact test. The cut-off value of HVPG for the efficacy assessment was calculated using a receiver operating characteristic (ROC) curve. A *P* value of 0.05 was regarded as significant. Excel Statistics 2015 software (SSRI Institute, Tokyo) was used for statistical analyses.

RESULTS

Patients

Among the 47 recruited patients, 7 were excluded from this prospective study: 4 met the exclusion criteria and 3 did not provide informed consent. Therefore, 40 patients were subjected to the clinical study and subsequent analysis. Patient characteristics are shown in Table 1. Patients consisted of 26 males (65.0%) and 14 females (35.0%), with the median age of 65 years (range, 40-82 years). The etiology of liver diseases was hepatitis C for 15 patients, hepatitis B for 3, alcoholic hepatitis for 15, primary biliary cirrhosis for 3, primary sclerosing cholangitis for 1, and non-alcoholic steatohepatitis for 3. According to the Child-Pugh classification, 3 patients were classified into class A, 19 into class B, and 18 into class C. Twelve patients had hepatocellular carcinoma. Twenty-five patients had esophageal varices, but did not require the treatment at the entry. Median serum albumin, sodium and creatinine levels were 2.6 (range, 1.6-3.7) g/dL, 139 (range, 124-146) mEq/L, 0.95 (range, 0.45-6.45) mg/dL, respectively. The median HVPG value and hyaluronic acid value were 240 (range, 105-580) mmH_2O and 420.7 (range, 122-6984), respectively. The median urinary osmotic pressure was 414.5 (range, 254-954) mOsm/L. The daily dosages of furosemide and spironolactone before the administration of tolvaptan were 37.0 ± 29.5 mg and 43.4 ± 26.8 mg, respectively.

Effects of tolvaptan, biochemical tests, and urinalysis

Changes in body weight and 24-h urine volume after the administration of tolvaptan are shown in Figure 1. Median 24-h urine volumes on days 1 and 7 were 1600 mL and 1582 mL, respectively. The median volume increases from the baseline were +492 mL ($P = 6.97 \times 10^{-5}$) and +474 mL ($P = 4.87 \times 10^{-4}$), respectively. The median body weight decreases from the baseline on days 1 and 7 were 1.0 kg ($P = 2.04 \times 10^{-6}$) and 1.3 kg ($P = 1.83 \times 10^{-5}$), respectively.

Patients were divided into two groups based on the median value of each baseline quantitative variable. Changes in body weight loss during 7 d were compared between the two groups (Figure 2A-G). Hyaluronic acid level was a marginally significant factor influencing the weight loss effect. Patients with lower hyaluronic acid level had favorable response to tolvaptan compared with those with higher hyaluronic acid level, though not significant ($P = 0.088$) (Figure 2G).

Association between HVPG and the effect of tolvaptan

Twelve patients rejected measurement of the HVPG. Therefore, the HVPG measurement procedures were performed in 28 patients before the administration of tolvaptan. Of these, 4 were excluded from subsequent analysis due to a hepatic vein-hepatic vein shunt on hepatic venography.

Patients were divided into two groups: those with HVPG of higher than 200 mmH_2O, which is the cutoff value for the diagnosis of portal hypertension and those with HVPG of 200 mmH_2O or lower. The median changes in body weight loss on day 7 were -0.2 kg in the former and -3.05 kg in the latter ($P = 0.012$) (Figure 2H). Using the ROC curve, the cutoff value of 190 mmH_2O (sensitivity: 75.0%, specificity: 93.3%, area under the curve: 0.825) was the most useful in discriminating responders from non-responders (Figure 3). Among patients with HVPG of 190 mmH_2O or lower,

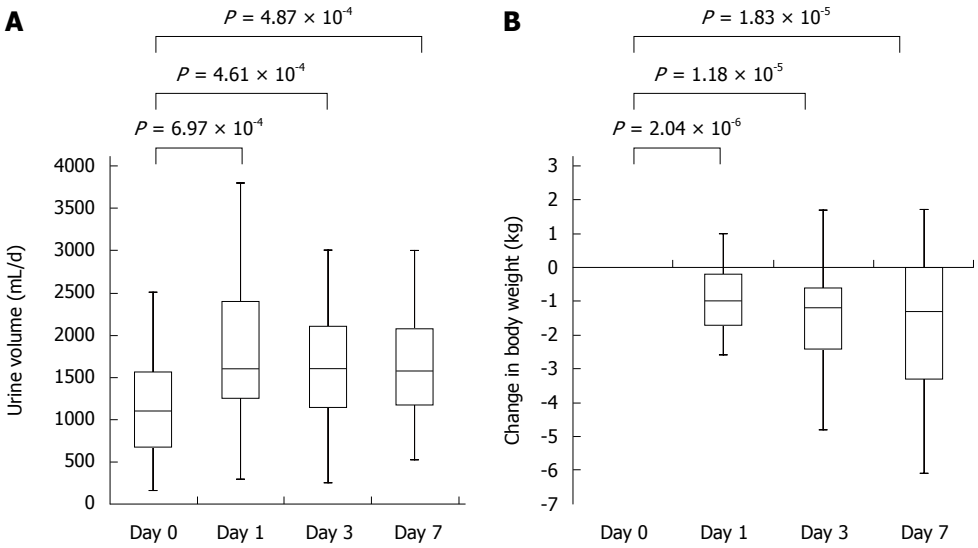
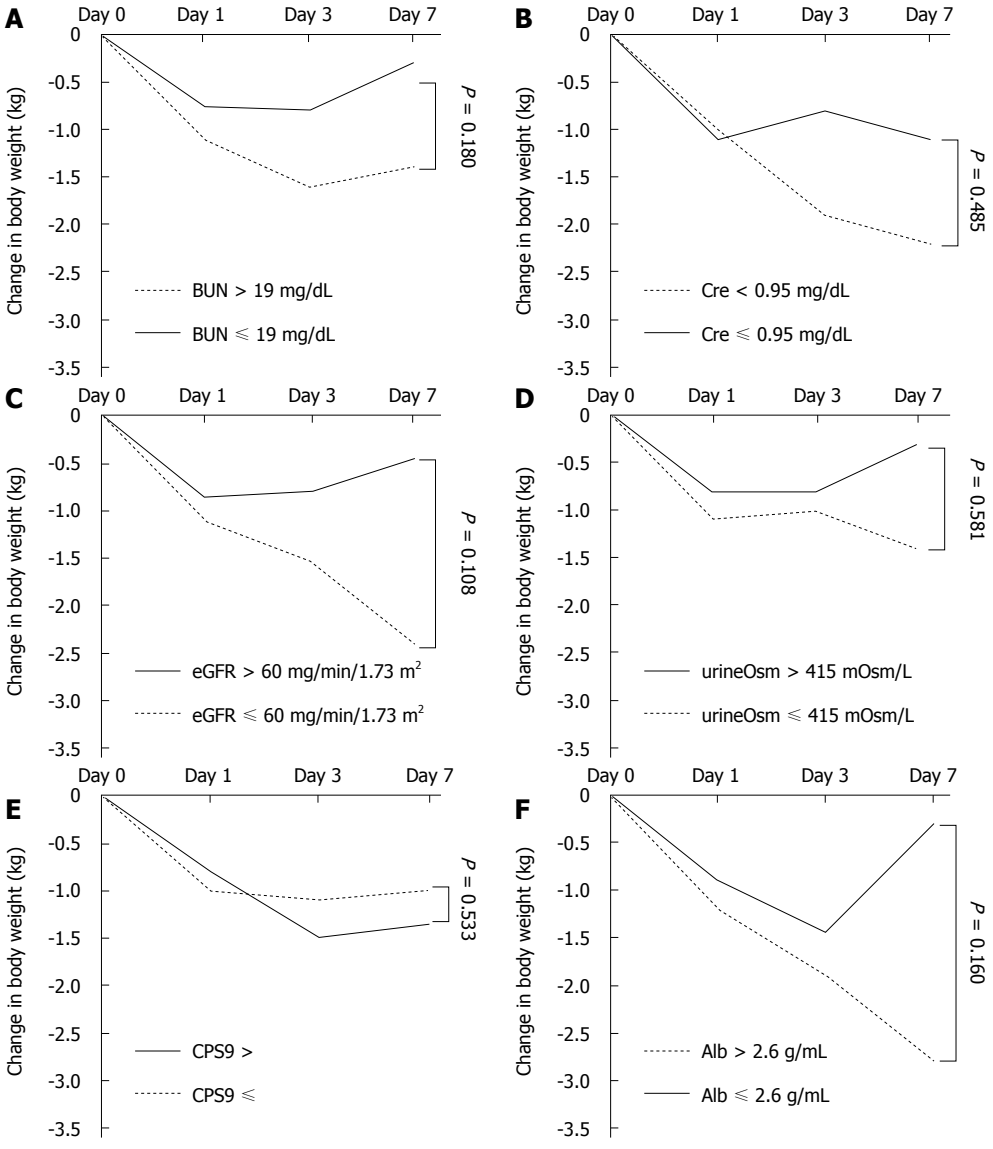


Figure 1 Effects of tolvaptan on liquid retention in all of the patients included in this study. A: Box and whisker plots of daily urine volumes during the first week of tolvaptan administration in all of the patients. Median values were 1108 mL, 1600 mL, 1500 mL and 1582 mL on day 0, 1, 3 and 7, respectively; B: Box and whisker plots of changes in body weight from baseline during the first week of tolvaptan administration in all of the patients. Median changes in body weight were -1 kg, -1.2 kg, and -1.3 kg on day 1, 3 and 7, respectively. Difference between day 0 (= baseline) and day1, 3 and 7 were compared by using Wilcoxon signed rank test.



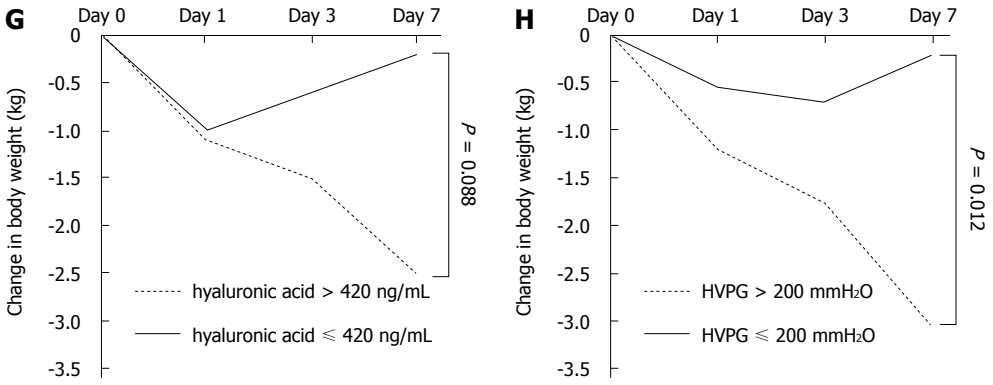


Figure 2 Change in body weight from baseline on each baseline factor during the first week of tolvaptan administration. Data are expressed as median. Patients were divided into two groups using the median value of each baseline variable: (A) serum BUN; (B) serum creatinine; (C) serum eGFR; (D) urine osmolality; (E) Child-Pugh Score (CPS); (F) serum albumin (Alb); (G) serum hyaluronic acid; and (H) Hepatic venous pressure gradient (HVPG).

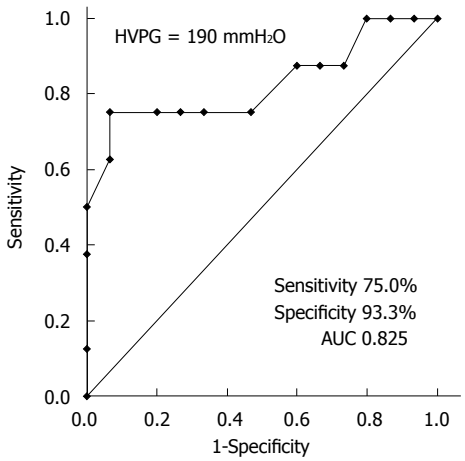


Figure 3 Optimal cutoff value of hepatic venous pressure gradient of the efficacy assessment was determined using ROC curve. The value of 190 mmH2O [sensitivity, 75.0%; specificity, 93.3%; and area under the curve (AUC), 0.825] was the most useful in predicting treatment response, defined as body weight loss of 2 kg or greater from the baseline. HVPG: Hepatic venous pressure gradient.

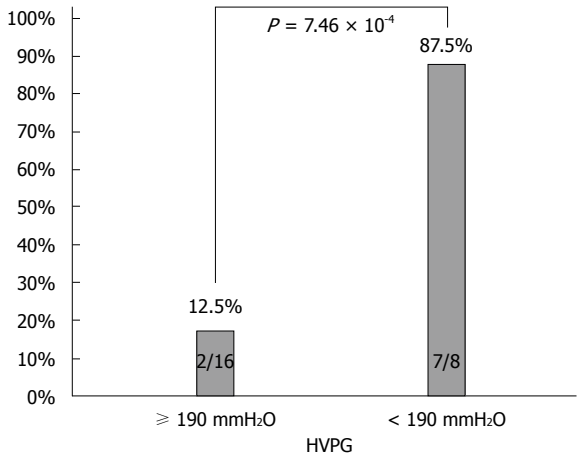


Figure 4 Difference in treatment response rates between patients with low and high hepatic venous pressure gradient. The response rate of 87.5% in the latter with 190 mmH2O or greater was significantly higher than that of 12.5% in the former with less than 190 mmH2O (P = 7.46 × 10^{-4}). HVPG: Hepatic venous pressure gradient.

7 of 8 patients (87.5%) were responders. By contrast, among those with HVPG of higher than 190 mmH2O, only 2 of 16 patients (12.5%) were responders (P = 7.46 × 10^{-4}) (Figure 4).

To examine the influence of tolvaptan on portal vein pressure, changes in HVPG after the administration of tolvaptan were evaluated in 19 patients, in whom the post-treatment HVPG was measured. HVPG values prior to and after the treatment were 213 (range, 105-305) and 210 (range, 150-340) mmH2O, respectively (not significant, P = 0.938, Figure 5A). Even when patients were sub-divided into two groups: those with HVPG of 190 mmH2O and lower (n = 7) and higher than 190 mmH2O (n = 12), no significant changes in HVPG prior to and after the treatment were observed in both subgroups (P = 0.108 and 0.684, respectively; Figure 5B and C).

Differences in background factor according to responses for tolvaptan

Next, based on previous clinical studies using tolvaptan, patients with a body weight decrease of 2 kg or greater from the baseline were regarded as responders. On the other hand, patients with decreases of less than 2 kg or increases from the baseline were regarded as non-responders. We analyzed differences in background factor according to responses for tolvaptan. HVPG (P = 0.045) and serum hyaluronic acid (P = 0.017) were detected as useful factors. All other characteristics factors did not have the significant difference between both groups (Table 2).

Safety

Adverse events were observed in 13 of 40 subjects (32.5%). The most frequent adverse event was pollakiuria, which occurred in six patients (15.0%).

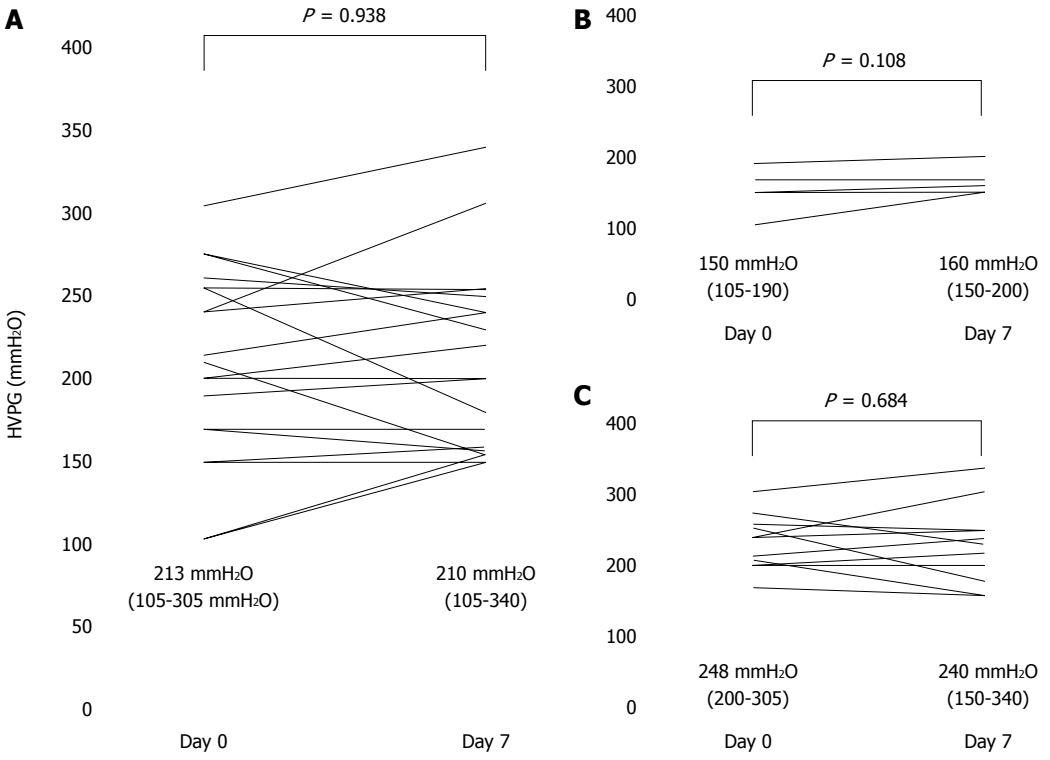


Figure 5 Changes in hepatic venous pressure gradient levels at day 0 and 7. Data are expressed as median (range in parenthesis). A: Overall patients (*n* = 19); B: Patients (*n* = 7) with low HVPG (≤ 190 mmH2O); C: Patients (*n* = 12) with high HVPG (> 190 mmH2O). There was not significant difference in any groups, indicating that tolvaptan had little impact on HVPG. HVPG: Hepatic venous pressure gradient.

Table 2 Comparison of demographic and clinical characteristics at baseline between responders and non-responders

Characteristics	Responder (*n* = 17)	Non-responder (*n* = 23)	*P* value
Age (yr)	66 (45-80)	61 (40-82)	0.8137
Body weight (kg)	62.3 (44.8-88.5)	61.7 (50.3-79.2)	0.7857
Liver disease etiology (Hepatitis B/Hepatitis C/Alcohol/PBC/PSC/NASH)	3/5/5/2/1/1	0/10/10/1/0/2	-
Child-Pugh classification (A-B/C)	9/8	13/10	1.000
Total bilirubin (mg/dL)	0.8 (0.4-11.4)	1.3 (0.5-26.2)	0.332
Serum albumin (g/dL)	2.5 (1.9-3.5)	2.6 (1.6-3.7)	0.733
Serum creatinine (mg/dL)	0.91 (0.62-1.83)	1.1 (0.45-6.45)	0.480
Serum eGFR (mL/min/1.73 m^2)	62 (24-85)	51 (8-112)	0.290
Serum sodium (mEq/L)	137.5 (125-146)	140 (124-144)	0.855
Serum hyaluronic acid (ng/mL)	335 (181-2843)	567.9 (122-6984)	0.017
BUN (mg/dL)	18.1 (8.1-81)	20.9 (10.1-81.8)	0.211
Urine osmolality (mOsm/L)	418 (257-700)	361.5 (254-954)	0.293
Hepatic venous pressure gradient (mmH2O)[1]	170 (105-580)	255 (150-350)	0.045
Hepatocellular carcinoma (with/without)	6/11	6/17	0.729
Esophageal varix (with/without)	10/7	15/8	0.518

[1]Hepatic venous pressure gradient was measured in 24 patients. Categorical variables are given as number. Continuous variables are given as median (range). BUN: Blood urea nitrogen; PBC: Pimary biliary cirrhosis; PSC: Primary sclerosing cholangitis; NASH: Nonalcoholic steatohepatitis.

Thirst was noted in five patients. Malaise was observed in two patients. Serum creatinine levels increased in three patients: one of them discontinued tolvaptan after 5 d of treatment [serum creatinine = 2.34 mg/dL (+1.59 mg/dL from baseline)] and recovered rapidly after the cessation. No other severe adverse events were noted.

DISCUSSION

Tolvaptan was approved as a drug for heart failure in Japan in 2010. Thereafter, its favorable therapeutic effects have been reported[22]. Furthermore, a phase Ⅲ study of tolvaptan for liquid retention was conducted in Japan. Sakaida *et al*[23] reported that body weight decreased by 1.95 kg and 24-h urine volume increased by 633 mL during a 7-d administration period, suggesting the efficacy and safety of tolvaptan in the treatment of liquid retention. In response to the encouraging data, tolvaptan is clinically available in Japan since 2013. In some patients, however, tolvaptan does not improve liquid retention. Little is known about the characteristics of patients who respond well to tolvaptan and the factors influencing the therapeutic effect. Furthermore, the role of tolvaptan in the therapeutic strategy for liquid retention currently remains unclear: whether tolvaptan is used separately from or in combination with conventional diuretics should be determined, and the commencing time of

tolvaptan needs to be clarified.

The present study is the first to show that response to tolvaptan correlated closely with HVPG, which reflects portal vein pressure in cirrhotic patients. Measurement of HVPG makes it possible to estimate the stage of liver fibrosis regardless of disease etiology[24,25], and to assess the severity and prognosis of liver cirrhosis[26,27] and the risk of complications, such as the rupture of esophageal varices, ascites, hepatic encephalopathy, and hepatorenal syndrome[28,29]. Ripoll *et al*[30] reported that decompensated liver cirrhosis was more likely to deteriorate in patients with HVPG of 10.0 mmHg (approximately 136 mmH_2O) or higher. Kumar *et al*[31] found that HVPG of 13.0 mmHg (approximately 177 mmH_2O) or higher was predictive of advanced fibrosis. In the present study, the cut-off value of 190 mmH_2O was the most useful in predicting treatment response, suggesting that tolvaptan exerts its effects on conventional diuretic-resistant patients with lower HVPG. For those with higher HVPG, combination with other treatments, such as TIPS, may be needed to improve tolvaptan-resistant liquid retention. However, HVPG was not decreased even in responders, indicating that tolvaptan has little impact on portal vein pressure. This phenomenon may be attributed to the antagonistic action site of tolvaptan, a vasopressin V_2 receptor, which is in the uriniferous tubules of the kidney alone, and thus does not cause vasoconstriction[12]. In other words, tolvaptan has no anti-vasoconstrictive effect on splanchnic vessels. By contrast, terlipressin, which acts on the vasopressin V_1 receptor, has vasoconstrictive effects on the visceral vessels and consequently reduces portal blood flow[32].

The direct relationship between high portal vein pressure and low responsiveness to tolvaptan is unclear. We examined the correlations between HVPG and various biochemical data in the present study. Although no significant factor could be found, low serum albumin and low eGFR levels might be associated with relatively high HVPG (data not shown). These variables reflect the reserved function of the liver and kidneys. Such patients with impaired liver and kidney functions are likely to have high HVPG, which attenuates the effect of tolvaptan.

The limitations of this study included the small number of patients examined and variations in the etiology of liver diseases. Only the short-term effect of tolvaptan was evaluated, water restriction and water intake were not measured, and drinking-related changes in body fluid volumes were not accurately assessed. However, a response criterion used in the present study (body weight loss of 2 kg or greater) may be appropriate, because the change in body weight after the administration of tolvaptan was reported to correlate with that in ascites volume[33]. Akiyama *et al*[34] administered tolvaptan for 42 d to patients: the initial significant effects lasted during the treatment period, though the mid- to long-term effects of tolvaptan remain controversial. A large-scale clinical trial has not yet been conducted, and it remains unknown whether tolvaptan improves the prognosis of patients with liquid retention.

Since hepatic venous catheterization to measure HVPG is relatively invasive, simple non-invasive tests and biomarkers are required. Previous studies reported that hepatic/splenic stiffness on transient elastography correlated with HVPG[35,36], whereas others indicated that the portal blood flow velocity and intrahepatic passage time measured using contrast-enhanced ultrasonography reflected severe portal hypertension[37]. However, these examinations are not useful in decompensated liver cirrhosis patients with ascites[38]. A recent study reported that the ICG value at 15 min[39] and inflammatory biomarkers, such as IL-1β and VCAM-1, correlated with portal blood pressure[40], though these studies involved only patients with compensated liver cirrhosis. A previous study showed that von Willebrand Factor antigen correlated with HVPG in decompensated liver cirrhosis patients with HVPG of 12 mmHg (approximately 163 mmH_2O) or more[41], though this test is not clinically available. Further studies are needed to develop an easy-to-implement, non-invasive method that sufficiently reflects HVPG even in patients with decompensated liver cirrhosis and predicts the therapeutic effects of tolvaptan.

The present study showed that responders to tolvaptan were likely to have lower HVPG and that tolvaptan had little impact on portal vein pressure. If high portal vein pressure in non-responders is decreased by beta-blocker, splenic artery embolization or TIPS, which could reduce portal vein pressure[42-46], the effect of tolvaptan may be improved. Additive or synergistic effects on liquid retention may be produced by lowering portal vein pressure in combination with these treatments.

In conclusion, the present study suggests that tolvaptan is effective for liquid retention in decompensated liver cirrhosis patients with lower portal vein pressure. By contrast, patients with higher HVPG have the likelihood of treatment failure. In the future, therapeutic strategy needs to be established to treat liquid retention in refractory patients.

ACKNOWLEDGMENTS

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COMMENTS

Background

A V_2 receptor antagonist became clinically available in Japan in 2013 for the treatment of liquid retention. On the other hand, factors influencing treatment response have not yet been elucidated. The authors conducted this prospective study to clarify such factors in 40 patients with decompensated liver cirrhosis complicated by liquid retention.

Research frontiers

Changes from the baseline in body weight were -1.0 kg and -1.3 kg on days 1 and 3, respectively. Hepatic venous pressure gradient (HVPG) was only significant factor influencing the weight loss effect of tolvaptan. When patients with body weight loss of 2 kg or greater from the baseline were defined as responders, the response rate was 87.5% (7/8) in patients with HVPG of 190 mmH_2O or less, whereas it was only 12.5% (2/16) in those with HVPG of greater than 190 mmH_2O.

Innovations and breakthroughs

At the initiation of tolvaptan treatment, the HVPG value, which was estimated from portal vein pressure, was measured using hepatic venous catheterization. The authors analyzed factors influencing the effects of tolvaptan including HVPG.

Applications

Since hepatic venous catheterization to measure HVPG is relatively invasive, simple non-invasive tests and biomarkers are required.

Terminology

The present study suggests that tolvaptan in the treatment of liquid retention is more effective for patients with lower portal vein pressure. On the other hand, patients with high portal vein pressure need to be treated by beta-blocker, splenic artery embolization or TIPS, which may reduce portal vein pressure.

Peer-review

The paper is devoted to the analysis of the efficacy of a V_2 antagonist used in heart failure and hyponatemia in cirrhotic patients with severe liquid retention.

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ORIGINAL ARTICLE

Randomized Controlled Trial

Suppository naproxen reduces incidence and severity of post-endoscopic retrograde cholangiopancreatography pancreatitis: Randomized controlled trial

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Author contributions: Mansour-Ghanaei F and Joukar F designed the study; Mansour-Ghanaei F, Taherzadeh Z and Sokhanvar H collected data; Joukar F contributed to data analysis; Joukar F and Sokhanvar H supervised the procedure; Mansour-Ghanaei F, Joukar F and Hasandokht T drafted the manuscript; all authors read and approved the final manuscript.

Institutional review board statement: The study was reviewed and approved by the ethics committee of the Gastrointestinal and Liver Diseases Research Center of Guilan University of Medical Science.

Clinical trial registration statement: This study is registered at Iranian Registry of Clinical Trials. The registration identification number is IRCT201105301155N14.

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

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Abstract

AIM: To determine the efficacy of rectally administered naproxen for the prevention of post-endoscopic retrograde cholangiopancreatography (ERCP) pancreatitis (PEP).

METHODS: This double-blind randomized control trial conducted from January 2013 to April 2014 at the Gastrointestinal and Liver Diseases Research Center in Rasht, Iran. A total of 324 patients were selected from candidates for diagnostic or therapeutic ERCP by using the simple sampling method. Patients received a single dose of Naproxen (500 mg; n = 162) or a placebo (n = 162) per rectum immediately before ERCP. The overall incidence of PEP, incidence of mild to severe PEP, serum amylase levels and adverse effects were measured. The primary outcome measure was the development of pancreatitis onset of pain in the upper abdomen and elevation of the serum amylase level to > 3 × the upper normal limit (60-100 IU/L) within 24 h after ERCP. The severity of PEP was classified according to the duration of therapeutic intervention for PEP: mild, 2-3 d; moderate 4-10 d; and severe, > 10 d

and/or necessitated surgical or intensive treatment, or contributed to death.

RESULTS: PEP occurred in 12% (40/324) of participants, and was significantly more frequent in the placebo group compared to the naproxen group ($P < 0.01$). Of the participants, 25.9% (84/324) developed hyperamylasemia within 2 h of procedure completion, among whom only 35 cases belonged to the naproxen group ($P < 0.01$). The incidence of PEP was significantly higher in female sex, in patients receiving pancreatic duct injection, more than 3 times pancreatic duct cannulations, and ERCP duration more than 40 min (Ps < 0.01). There were no statistically significant differences between the groups regarding the procedures or factors that might increase the risk of PEP, sphincterotomy, precut requirement, biliary duct injection and number of pancreatic duct cannulations. In the subgroup of patients with pancreatic duct injection, the rate of pancreatitis in the naproxen group was significantly lower than that in the placebo (6 patients *vs* 23 patients, $P < 0.01$, RRR = 12%, AR = 0.3, 95%CI: 0.2-0.6). Naproxen reduced the PEP in patients with $\geqslant$ 3 pancreatic cannulations ($P < 0.01$, RRR = 25%, AR = 0.1, 95%CI: 0.1-0.4) and an ERCP duration > 40 min ($P < 0.01$, RRR = 20%, AR = 0.9, 95%CI: 0.4-1.2).

CONCLUSION: Single dose of suppository naproxen administered immediately before ERCP reduces the incidence of PEP.

Key words: Naproxen; Nonsteroidal anti-inflammatory drugs; Pancreatic duct injection; Post-endoscopic retrograde cholangiopancreatography; Pancreatitis; Serum amylase

Core tip: Acute pancreatitis is the most common serious complication of endoscopic retrograde cholangiopancreatography (ERCP); prevention of post-ERCP pancreatitis (PEP) has become more challenging. The use of nonsteroidal anti-inflammatory drugs is effective in this condition. This study evaluated the efficacy of rectally administered naproxen for the prevention of PEP in composition with placebo immediately before ERCP.

Mansour-Ghanaei F, Joukar F, Taherzadeh Z, Sokhanvar H, Hasandokht T. Suppository naproxen reduces incidence and severity of post-endoscopic retrograde cholangiopancreatography pancreatitis: Randomized controlled trial. *World J Gastroenterol* 2016; 22(21): 5114-5121 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i21/5114.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i21.5114

INTRODUCTION

Acute pancreatitis is the most common complication of endoscopic retrograde cholangiopancreatography (ERCP), with an incidence rate of 1%-10% that can reach 40% or more, depending on the presence of risk factors[1-5]. Factors predicting post-ERCP pancreatitis (PEP) include young age, female sex, pancreas divisum, sphincter of Oddi dysfunction, previous ERCP-induced pancreatitis, multiple attempts for pancreatic duct cannulation, and pancreatic duct injection[6,7]. Although most cases of PEP are clinically mild or moderate in severity, 10% present severe manifestations[8,9].

ERCP complications may be minimized by reducing pancreatic secretion, interrupting inflammatory cascades, relaxing the sphincter of Oddi, and preventing intra-acinar trypsinogen activation infection[10,11]. Several pharmacologic agents, including octreotide[12], diclofenac[13,14], and recombinant interleukin-10[15,16], have been investigated to reduce the incidence and severity of PEP. Additionally, the protease inhibitors gabexate mesylate[17], and somatostatin[18,19] had been shown are effective in preventing PEP, particularly when administered as an intra-venous infusion[20,21].

Evidence suggests that the patient's inflammatory response to pancreatic duct imaging and instrumentation contribute to the development of PEP[7,22-27]. As phospholipase A_2 may play a vital role in the initial inflammatory cascade of acute pancreatitis[24], identifying pharmacologic agents that inhibit or disturb this cascade may prevent or limit the pancreatitis and its consequences. In some randomized controlled trials, various Oral and suppository of nonsteroidal anti-inflammatory drugs (NSAIDs) have shown promising prophylactic activity with regard to PEP[13,14,28-30]. However, different result were seen about the effectiveness of the intraduodenal indomethacin[30,31]. NSAIDs are easily administered, inexpensive, and relatively safe when given as a single dose, making them an attractive treatment option. Despite these benefits of NSAIDs and findings of the recent meta-analysis[32,33] indicated that rectal diclofenac or indomethacin reduce the incidence and severity of PEP, results of the several studies appear to contradict these conclusions[10,34-36]. Tilak Shah *et al*[35] mentioned several questions in this area; comparison between various NSAID, higher dose of drug and different rout of administration. Additionally, there are some study[37,38] reported NSAIDs can cause acute pancreatitis with the highest risk for diclofenac (OR = 5.0, 95%CI: 4.2-5.9) and the lowest for naproxen (OR = 1.1, 95%CI: 0.7-1.7)[39]. Therefore, we conducted a double-blind, randomized, and controlled clinical trial to evaluate the prophylactic effect of a naproxen suppository for the prevention of PEP.

MATERIALS AND METHODS

Study population

Participants were serially enrolled as they were seen for diagnostic or therapeutic ERCP at the gastroenterology ward of Razi Hospital, a referral center in Rasht, Iran between January 2013 and April 2014. Patients over 18 years of age who were scheduled to undergo ERCP and willing to provide written informed consent for study participation were included. Patients who had acute or active pancreatitis, a history of chronic pancreatitis and/or previous endoscopic sphincterotomy, active peptic ulcer disease, rectal disease, aspirin-induced asthma, use of NSAIDs during the preceding two weeks, hypersensitivity to NSAIDs, renal dysfunction, or were pregnant or breastfeeding, were ineligible for the study.

Study design

The protocol for this randomized, controlled clinical trial (IRCT201105301155N14) was approved by the ethics committee of the Gastrointestinal and Liver Diseases Research Center of Guilan University of Medical Science, and written informed consent (per the Helsinki declaration) was obtained from each participant. Eligible participants (n = 324) were selected from candidates for diagnostic or therapeutic ERCP by using the simple sampling method. The sample size was based on the frequency of pancreatitis in the placebo group (P_1 = 0.1)[28] compared to the study drug group (P_2 = 0.04) with α = 0.05 and β = 20%. Selected patients were randomly assigned using permuted-block randomization to receive either a naproxen (500 mg; Behvazan Pharmacy Co., Tehran, Iran) (n = 162) or placebo (n = 162) suppository immediately before ERCP. Concealed envelopes with naproxen or placebo, which appeared identical, were dispensed in sequence. Study participants, ERCP physicians, and nurses who administered treatment were unaware of the nature of the drugs. The group assignment was only known by the programmer of the database used during the study.

ERCP was performed by using a standard therapeutic duodenoscope (Olympus CO.) with the patient under local anesthesia with 2% lidocaine and after premedication by intravenous administration of 0.05 mg/kg of Midazolam or in cases with contraindication, intravenous administration 1 mg/kg pethidine. Blood pressure, heart rate, and oxygen saturation were monitored with automated devices. Contrast medium (Meglumine Compound 76%) was injected manually, under fluoroscopic guidance. ERCPs were carried out by 3 experienced endoscopists, with a mean number of sphincterotomy procedures performed of about 5 to 7 per week.

Outcome and data measurements

The primary outcome measure was the development of pancreatitis, defined according to the guideline of Cotton *et al*[7] as onset of pain in the upper abdomen and elevation of the serum amylase level to > 3 × the upper normal limit (60-100 IU/L) within 24 h after ERCP. The severity of PEP was classified according to the duration of therapeutic intervention for PEP: mild, 2-3 d; moderate 4-10 d; and severe, > 10 d and/or necessitated surgical or intensive treatment, or contributed to death[7].

Serum amylase was measured before, 2 h after, and any time the patients complained of pain within 24 h after ERCP; otherwise, it was routinely measured 24 h after ERCP. After 2 h, patients with normal serum amylase or no history of abdominal pain, nausea and vomiting were permitted to resume oral intake. All patients with prolonged pancreatitis symptoms (> 48 h) were assessed for complications of pancreatitis (abscess, pseudocyst, or fluid collection) by CT.

Demographic characteristics, risk factors, ERCP procedural elements, and follow-up data were collected at the time of the procedure and 24 h after ERCP by a trained physician who was unaware of study-group assignments. ERCP duration, the number of biliary and pancreatic cannulations, findings of the biliary and/or pancreatic duct, and interventions such as sphincterotomy, papillary balloon dilation, and stenting were recorded.

Table 1 Patient characteristics *n* (%)

Characteristic	Naproxen (n = 162)	Placebo (n = 162)
Age, yr	46.3 ± 8.3	44.7 ± 9.7
Female	78 (48.1)	73 (45.1)
Pancreatitis severity		
Mild	8 (20.0)[b]	18 (45.0)
Moderate	4 (10.0)[b]	10 (25.0)
Severe	0 (0)[b]	0 (0)

[b]P < 0.01 *vs* Placebo. Data are presented as mean ± SD or n (%).

Statistical analysis

A two-tailed χ^2 test was used to analyze the difference in the proportion of patients with PEP in the naproxen and placebo groups. Data are expressed as odds ratio (OR) with 95% confidence interval (CI). Additional exploratory subgroup analyses were performed according to age, sex, and procedure, and are reported as relative risk (RR), absolute risk (AR) and relative risk reduction (RRR). All comparisons were carried out on a two-tailed basis. Statistical analysis was carried out with the SPSS (version 16) and P < 0.05 was considered statistically significant. Ninety-five percent significant intervals (CI) for the proportions were calculated.

RESULTS

There were 78 (48.1%) women in the naproxen group and 73 (45.1) women in the control group. The mean age ± SD of the patients in the intervention group was

Table 2 Incidence of post-endoscopic retrograde cholangio-pancreatography pancreatitis

Variable	Naproxen[1]	Placebo[2]
Sex		
Female	9/12[b]	19/28
Male	3/12	9/28
Age (yr)		
< 40	5/12	10/28
> 40	7/12	18/28
Sphincterotomy		
Yes	8/129	23/119
No	4/33	5/43
Precut required	2/31	2/31
Pancreatic duct injection		
Yes	6/75[b]	23/84
No	6/82	5/83
Pancreatic duct cannulations		
⩾ 3	2/6[b]	10/23
⩽ 2	3/6	14/23
ERCP duration (min)		
> 40	4/12[b]	12/28
< 40	8/12	16/28
Biliary duct injection		
Yes	9/134	24/135
No	3/23	4/25

[1]Pancreatitis/naproxen (12/162, 7.4%); [2]Pancreatitis/placebo (28/162, 17%). [b]$P < 0.01$ *vs* Placebo.

46.3 ± 8.3, and in the control group it was 44.7 ± 9.7. The characteristics of trial participants are presented in Table 1. PEP occurred in 12% (40/324) of participants, and was significantly more frequent in the placebo group (28/162, 17%) compared to the naproxen group (12/162, 7.4%) ($P < 0.01$). Of the participants, 25.9% (84/324) developed hyperamylasemia within 2 h of procedure completion, among whom only 35 cases belonged to the naproxen group ($P < 0.01$).

Analyses in different group indicated that the incidence of PEP was significantly higher in patients receiving pancreatic duct injection, cases with 3 times or more pancreatic duct cannulations, ERCP duration > 40 min and female sex (*P*s < 0.01) (Table 2). Logistic regression analysis of possible risk factors for PEP indicated that pancreatic duct injection, duration of ERCP, female sex and age were significant risk factors (*P*s < 0.05) (Table 3). There were no statistically significant differences between the groups regarding the procedures or factors that might increase the risk of PEP, including, sphincterotomy, precut requirement and biliary duct injection.

In the subgroup of patients with pancreatic duct injection, the rate of pancreatitis in the naproxen group was significantly lower than that in the placebo group (6 patients *vs* 23 patients; $P < 0.01$, RRR = 12%, AR = 0.3, 95%CI: 0.2-0.6). Naproxen reduced the PEP in patients with ⩾ 3 pancreatic cannulations ($P < 0.01$, RRR = 25%, AR = 0.1, 95%CI: 0.1-0.4) and an ERCP duration > 40 min ($P < 0.01$, RRR = 20%, AR = 0.9, 95%CI: 0.4-1.2).

The most common final diagnosis after ERCP was choledocholithiasis, followed by several cases of sphincter of Oddi dysfunction, common bile duct tumors, and choledochal cysts (Figure 1). We did use pancreatic duct stenting in the nobody of study subjects. All patients were discharged in good health and there were no reported side effects.

DISCUSSION

A systematic review of five clinical trials[39], as well as two subsequent meta-analyses[40,41], indicate that administration of NSAIDs significantly decreases the incidence of PEP. Our findings show that a single dose of suppository naproxen given immediately before ERCP significantly reduces the overall incidence and severity of PEP. Elmunzer *et al*[42] in a multicenter, randomized, placebo-controlled, double-blind clinical trial showed post-ERCP pancreatitis developed in 16.9% of placebo group *vs* 9.2% in the indomethacin group, as well as, indomethacin decrease the incidence of moderate to severe PEP. Andrade-Dávila *et al*[43] conducted a controlled clinical trial where patients at least one major and/or two minor risk factors for developing post-ERCP pancreatitis. They suggested rectal indomethacin reduced the incidence of post-ERCP pancreatitis among patients at high risk of developing this complication. In our randomized controlled trial, the number of patients who would need to be treated to prevent one episode of pancreatitis was 10. Sethi *et al*[44] in a meta analysis concluded rectal NSAIDs can decrease PEP with 11 patients needed to treatment. However, another meta-analysis showed the number needed to treat was 17[30]. On the other hand, recent controlled clinical trial a number needed to treat of 6.5 patients were calculated to prevent an episode of post-ERCP pancreatitis[43].

The occurrence of PEP varies according to patient characteristics and the type of intervention performed. We found that pancreatic duct injection, duration of ERCP (> 40 min), female sex and age (< 40 year) were significant risk factors for developing PEP, consistent with other studies[9,14,25,42]. European Society of Gastrointestinal Endoscopy Guideline presented cannulation attempts duration > 10 min and younger age increase the incidence of PEP[29]. Similarly, Sotoudehmanesh *et al*[14] demonstrated the protective effect of indomethacin for PEP in the patients with pancreatic duct injection. Furthermore, Elmunzer *et al*[42] showed that indomethacin significantly reduced the risk of moderate to severe PEP from 16.1% to 9.7% in patients with pancreatic injections. In our study, the risk of PEP was not associated with undergoing sphincterotomy, having sphincter of Oddi dysfunction. These findings are in contrast to those of Murray *et al*[13] who found that diclofenac was protective in patients who had sphincterotomy and those without sphincter of Oddi hypertension. Furthermore, recent guideline updated in 2014[29], female gender presented

Table 3 Risk factors for post-endoscopic retrograde cholangiopancreatography pancreatitis

Variable	Pancreatitis, *n*		OR	RR (95%CI)	RRR (%)	AR (%)
	Yes	No				
Group						
Naproxen	12	150	0.4[b]	0.42 (0.22-0.81)	58	-138
Placebo	28	134				
Sex						
Female	28	123	3[b]	2.67 (1.40- 5.07)	167	62.54
Male	12	161				
Age (yr)						
< 40	15	63	2.4[b]	2.2 (1.22-3.96)	120	54.54
> 40	25	261				
Sphincterotomy						
Yes	31	217	1	1.05 (0.52-2.11)	5	4.76
No	9	67				
Pancreatic duct injection						
Yes	29	130	2.1[b]	1.88 (1.06-3.32)	88	46.81
No	16	149				
Pancreatic duct cannulations						
⩾ 3	12	37	2.1	1.87 (0.93-3.74)	87	46.52
⩽ 2	14	93				
ERCP duration (min)						
> 40	16	54	2.6[b]	2.30 (1.29-4.09)	130	56.52
< 40	24	218				
Biliary duct injection						
Yes	33	236	1.7	1.66 (0.76-3.63)	66	39.75
No	7	88				

[b]$P < 0.01$ *vs* Placebo. OR: Odds ratio; RR: Relative risk; RRR: Relative risk reduction; AR: Absolute risk.

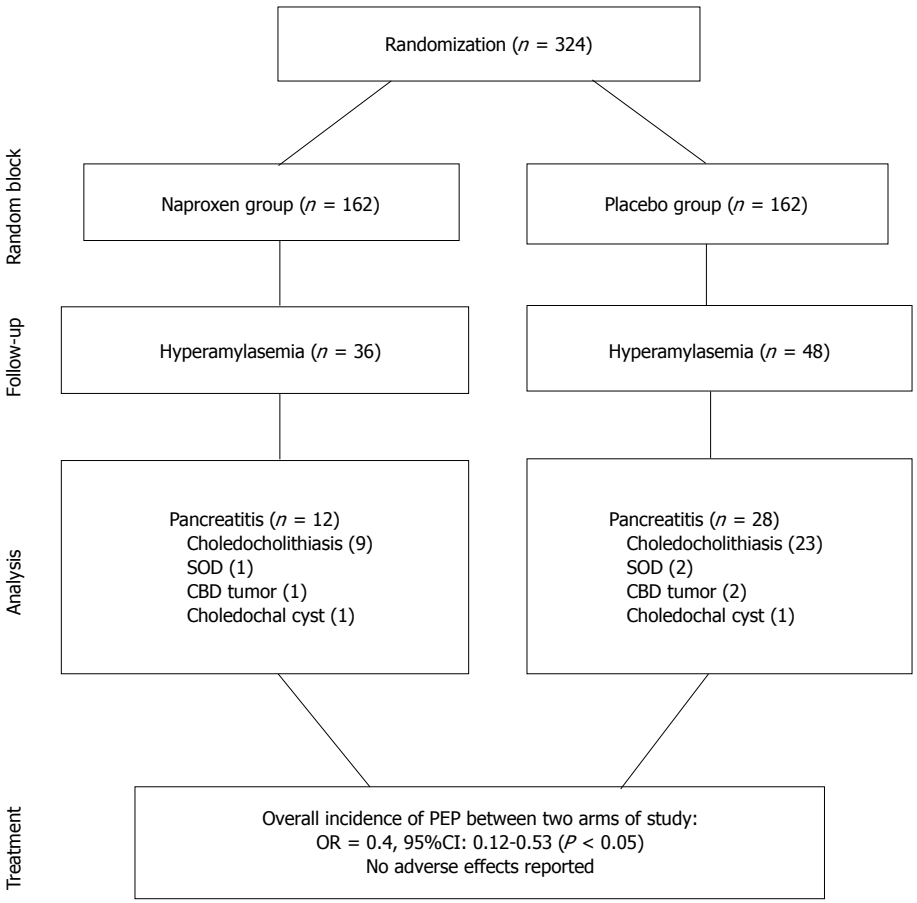

Figure 1 Participant selection. CBD: Common bile duct; PEP: Post-endoscopic retrograde cholangiopancreatography pancreatitis; SOD: Sphincter of Oddi dysfunction.

as a risk factor for PEP. In parallel our study, in recent controlled clinical trial, Suspected sphincter dysfunction oddi were not risk factor for PEP[43]. Till now, several meta-analyses[3,30,39-41,45] conclude that NSAIDs used in the different routes of administration decrease the incidence of pancreatitis and severity of pancreatitis. Although, the results of our study are relevant because the drug was rectally administrated immediately before procedure, the main difference between our study and those previously reported was the use of naproxen instead of indomethacin or diclofenac. Hence, to support the conclusion, a high-quality multicenter randomized clinical trial is required to better describe the effectiveness of naproxen as a NSAID.

In conclusion, a single-dose prophylactic naproxen suppository significantly decreases the occurrence and severity of PEP, particularly in those with pancreatic duct injections, multiple pancreatic duct cannulation attempts, those younger than 40 years of age, and requiring a procedure lasting more than 40 min. Moreover, this treatment produced no adverse events, consistent with previous works[10,28,40], and should therefore be administered immediately before ERCP procedures.

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COMMENTS

Background

Acute pancreatitis is the most feared complication of endoscopic retrograde cholangiopancreatography (ERCP) because it has the greatest potential for causing prolonged hospitalization, major morbidity, and occasionally death.

Research frontiers

Acute pancreatitis is the most common complication of ERCP; prevention of post-ERCP pancreatitis (PEP) has become more challenging.

Innovations and breakthroughs

Prevention of PEP has become more challenging. The use of nonsteroidal anti-inflammatory drugs (NSAIDS) is effective in this condition but selection the best effective drug is required more examination of it. This study is based on this real.

Applications

This study evaluated the efficacy of rectally administered Naproxen for the prevention of PEP in patients received a single dose of naproxen or a placebo immediately before ERCP.

Peer-review

This study provides useful information for prevention of PEP. The authors show that a single dose of suppository naproxen administered immediately before ERCP reduces the incidence and severity of PEP.

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META-ANALYSIS

Contemporary meta-analysis of short-term probiotic consumption on gastrointestinal transit

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Author contributions: Miller LE and Ouwehand AC designed the research; Miller LE and Zimmermann AK performed the research; Miller LE analyzed the data; Miller LE, Zimmermann AK, and Ouwehand AC wrote the paper; and Miller LE, Zimmermann AK, and Ouwehand AC approved the final draft of the paper.

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Conflict-of-interest statement: Miller LE is a consultant to Bio-K Plus, DuPont, Fonterra, Natren, and UAS Laboratories. Ouwehand AC is an employee of DuPont Nutrition and Health. Zimmermann AC denies any conflict of interest.

Data sharing statement: No additional data are available.

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Abstract

AIM: To determine the efficacy of probiotic supplementation on intestinal transit time (ITT) in adults and to identify factors that influence these outcomes.

METHODS: We conducted a systematic review of randomized controlled trials of probiotic supplementation that measured ITT in adults. Study quality was assessed using the Jadad scale. A random effects meta-analysis was performed with standardized mean difference (SMD) of ITT between probiotic and control groups as the primary outcome. Meta-regression and subgroup analyses examined the impact of moderator variables on SMD of ITT.

RESULTS: A total of 15 clinical trials with 17 treatment effects representing 675 subjects were included in this analysis. Probiotic supplementation was moderately efficacious in decreasing ITT compared to control, with an SMD of 0.38 (95%CI: 0.23-0.53, $P < 0.001$). Subgroup analyses demonstrated statistically greater reductions in ITT with probiotics in subjects with *vs* without constipation (SMD: 0.57 *vs* 0.22, $P < 0.01$) and in studies with high *vs* low study quality (SMD: 0.45 *vs* 0.00, $P = 0.01$). Constipation ($R^2 = 38\%$, $P < 0.01$), higher study quality ($R^2 = 31\%$, $P = 0.01$), older age ($R^2 = 27\%$, $P = 0.02$), higher percentage of female subjects ($R^2 = 26\%$, $P = 0.02$), and fewer probiotic strains ($R^2 = 20\%$, $P < 0.05$) were predictive of decreased ITT with probiotics in meta-regression. Medium to large treatment effects were identified with *B. lactis* HN019 (SMD: 0.67, $P < 0.001$) and *B. lactis* DN-173 010 (SMD: 0.54, $P < 0.01$) while other probiotic strains yielded negligible reductions in ITT relative to control.

CONCLUSION: Probiotic supplementation is moderately efficacious for reducing ITT in adults. Probiotics were most efficacious in constipated subjects, when evaluated in high-quality studies, and with certain probiotic strains.

Key words: Constipation; Gastrointestinal; Intestinal transit time; Meta-analysis; Probiotics

Core tip: We performed a contemporary systematic review and meta-analysis of randomized controlled trials to determine the effects of short-term probiotic supplementation on transit time in adults. Probiotic supplementation is moderately efficacious for reducing intestinal transit time in adults. Probiotics were most efficacious in constipated subjects, when evaluated in high-quality studies, and with certain probiotic strains.

Miller LE, Zimmermann AK, Ouwehand AC. Contemporary meta-analysis of short-term probiotic consumption on gastrointestinal transit. *World J Gastroenterol* 2016; 22(21): 5122-5131 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i21/5122.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i21.5122

INTRODUCTION

The human colonic microbiota is a complex ecosystem involved in maintenance of health and physiological functions of the host. Disturbances within the microbiota may result in gastrointestinal disorders such as constipation, irritable bowel syndrome, or periodic bouts of irregularity. Functional gastrointestinal disorders are a highly prevalent group of persistent and recurring conditions with a prevalence of 69% in the general population[1]. Slow intestinal transit is a common manifestation of functional gastrointestinal disorders affecting the bowel[2] and may also occasionally affect otherwise healthy individuals. Although the benefits of reducing intestinal transit time (ITT) in patients with constipation are obvious, reductions in ITT are also considered a beneficial physiological effect in the non-diseased general population[3]. Over-the-counter and prescription medications intended to normalize intestinal transit are widely utilized although no known treatment is considered efficacious, safe, and cost effective[4]. Probiotics are live micro-organisms that confer a health benefit on the host when administered in adequate dosages[5] and have been extensively studied for enhancement of gastrointestinal health[6,7]. Previously, we performed the first systematic review and meta-analysis on the efficacy of probiotic supplementation on ITT in adults[8]. The purpose of this study was to update these findings with data from randomized controlled trials (RCTs) published over the 3-year period since our last review.

MATERIALS AND METHODS

Literature search

This study was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA)[9]. We searched MEDLINE and EMBASE for RCTs of probiotic supplementation that reported ITT in adults by using a combination of relevant keywords. The details of the MEDLINE search strategy are listed in Table 1. The syntax for EMBASE was similar, but adapted as necessary. Additionally, manual searches were conducted using the Directory of Open Access Journals, Google Scholar, and the reference lists of included papers and other relevant meta-analyses. No date restrictions were applied to the searches. The final search was conducted in October 2015.

Table 1 MEDLINE search strategy

Therapeutic search terms
Probiotic
Synbiotic
Lactobacill
Bifidobacteri
Yogurt (yoghurt)
Fermented milk
Main outcome search terms
Gastrointestinal
Transit
Gut
Motility
Colonic
Constipation
Irritable bowel
Combination terms
or/1-6
or/7-13
and/14-15

Study selection

Two researchers independently selected studies for inclusion in the review. Disagreements were resolved by consensus. Titles and abstracts were initially screened to exclude manuscripts published in non-English journals. Next, review articles, commentaries, letters, and case reports were excluded. Lastly, we excluded studies of subjects where ITT reduction was undesirable or uninterpretable (*e.g.*, diarrhea or mixed IBS subtypes). Full-text of the remaining manuscripts was then retrieved and reviewed. Publications that failed to report ITT or that described non-randomized, non-controlled, or otherwise irrelevant studies were also excluded.

Data extraction

Data were extracted from eligible peer-reviewed articles by one author and then verified by a second author. Data extraction discrepancies between the two researchers were resolved by consensus. The following variables were recorded in a pre-designed database: general manuscript information (author, institution name and location, journal, year, volume, page numbers), study design characteristics (study quality, study design, sample size, method of ITT assessment,

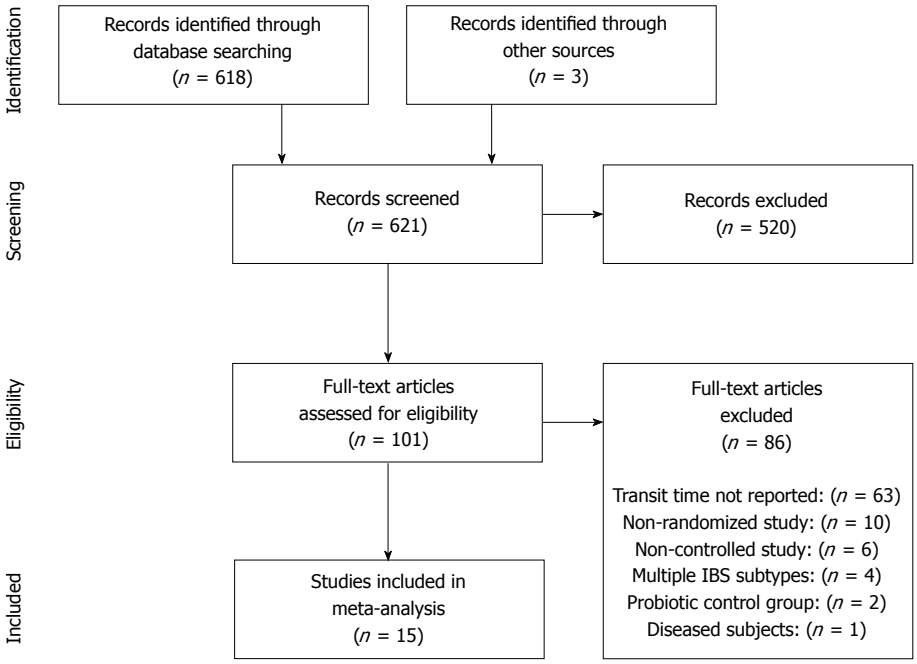

Figure 1 PRISMA flow diagram.

probiotic strain, daily dosage, product delivery method, and treatment duration), subject characteristics (age, gender, body mass index, and condition), and ITT summary statistics necessary for meta-analysis.

Quality assessment

The Jadad scale was used to assess RCT study quality[10]. Studies were scored according to the presence of three key methodological features: randomization, blinding and subject accountability. Randomization was scored from 0 to 2, blinding was scored from 0 to 2, and subject accountability was scored 0 or 1. RCTs with a score of 3 to 5 were classified as high quality; studies with a score of 0 to 2 were classified as low quality.

Statistical analysis

A random effects meta-analysis model was selected *a priori* based on the assumption that treatment effects were heterogeneous given the differences in probiotic strain, study design characteristics, and subject characteristics among studies. The standardized mean difference (SMD) and 95% confidence interval (CI) were the statistics of interest to describe treatment effects since different measures of ITT (*e.g.*, whole gut, colonic, oro-cecal, *etc*.) were utilized in the included studies. The SMD is calculated as the mean difference in ITT between probiotic and control groups divided by the pooled standard deviation in ITT. SMD values of 0.2, 0.5, and 0.8 are defined as small, medium, and large, respectively[11]. Positive SMDs imply that probiotics were more effective in reducing ITT *vs* control while negative SMDs imply a greater treatment effect with control *vs* probiotics. A forest plot was used to illustrate the individual study findings and the random effects meta-analysis results. Heterogeneity of effects across studies was estimated with the I^2 statistic where values of $\leqslant$ 25%, 50%, and $\geqslant$ 75% represent low, moderate, and high inconsistency, respectively[12]. In addition, a one study removed meta-analysis was performed to assess the influence of individual studies on the meta-analysis findings. Publication bias was visually assessed with a funnel plot and quantitatively assessed using Egger's test[13]. Meta-regression and subgroup analyses were performed to explore sources of heterogeneity. All analyses were performed using Comprehensive Meta-analysis (version 2.2, Biostat, Englewood NJ). The statistical methods of this study were reviewed by Clinton Hagen, MS (Mayo Clinic, Rochester, MN).

RESULTS

Study selection

Our initial database search retrieved 618 titles and abstracts; hand searching relevant bibliographies identified 3 additional records. After screening records for inclusion criteria, 101 full text articles were reviewed for eligibility. Ultimately, 15 RCTs with 17 treatment effects representing 675 unique subjects were included in the final analysis[14-28]. A flow chart of study identification and selection is shown in Figure 1.

Study characteristics

Sample sizes ranged from 10 to 36 per treatment arm for parallel groups designs (9 studies) and from 12 to 83 for cross-over designs (6 studies). Thirteen RCTs contributed one treatment effect each and two RCTs contributed two effects each; the study of Rosenfeldt

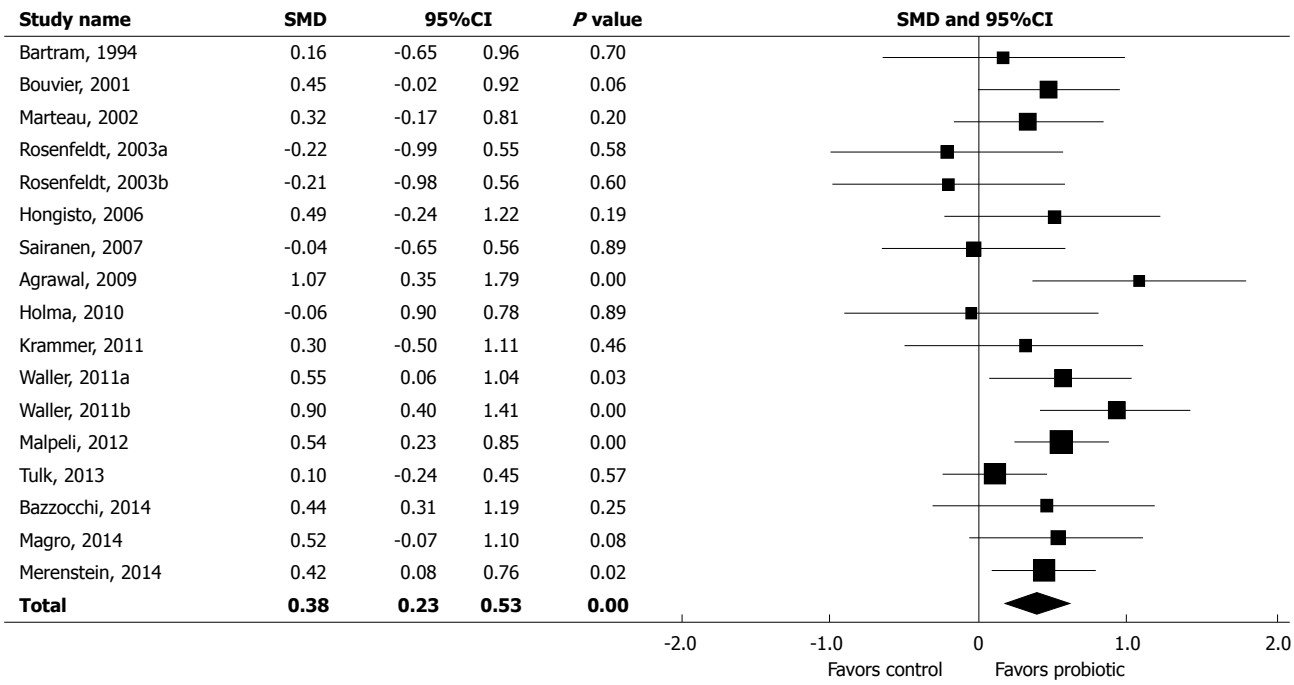

Figure 2 Forest plot of standardized mean difference in intestinal transit time across studies. Random effects model. I^2 = 20%, P = 0.22. SMD: Standardized mean difference.

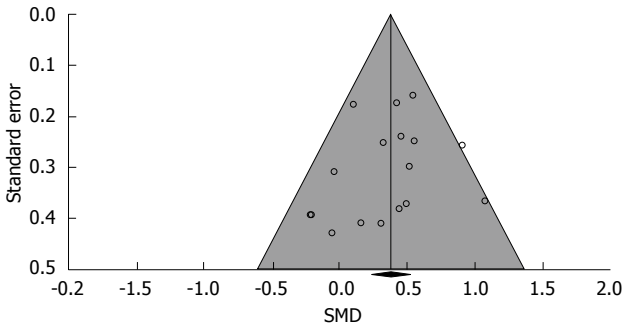

Figure 3 Funnel plot of standardized mean difference in intestinal transit time across studies. Egger's P value = 0.44 for publication bias. SMD: Standardized mean difference.

and colleagues[21] assessed two different probiotic formulations and the study of Waller and colleagues[23] assessed two different dosages of the same probiotic strain. Daily probiotic dosages varied considerably among studies, ranging from 5×10^8 to 9.8×10^{10} colony forming units (CFU) per day (median 1.6×10^{10} CFU per day). Probiotic treatment periods ranged from 10 to 28 d (median 18 d). Intestinal transit time was measured using radiopaque markers in 13 studies and with carmine red dye in 2 studies. The most commonly tested product format was yogurt or other forms of fermented milk. Six (40%) studies included other components in the active product known to influence ITT such as lactulose, psyllium, inulin, polydextrose, maltodextrose, and oligofructose (Table 2).

Subject characteristics

Nine treatment effects were calculated for subjects with constipation or IBS-C while 8 effects were based on healthy subjects. Subjects were predominantly female, mean age ranged from 23 to 50 years, and mean body mass index ranged from 19 to 32 kg/m^2 (Table 3).

Study quality assessment

Overall, the quality of RCT reporting was medium with a median Jadad score of 3 (range: 1-5). Twelve of 17 treatment effects were based on high quality (Jadad score 3-5) trials. The method of randomization was inadequately described in most studies. Descriptions of blinding were adequate overall. Subject accountability in RCTs was sufficiently detailed in 11 of 17 cases (Table 4).

Main results

In relation to controls, probiotic supplementation statistically decreased ITT, with an SMD of 0.38 (95%CI: 0.23-0.53, $P < 0.001$) (Figure 2). Only 5 of 17 treatment effects statistically favored probiotic supplementation. There was low heterogeneity among studies (I^2 = 20%, P = 0.22) with no evidence of publication bias (Egger's regression test: P = 0.44) (Figure 3). A one study removed sensitivity analysis was performed to determine the influence of individual studies on main outcomes. Overall, no single study significantly influenced the observed SMD of ITT with probiotics *vs* control. SMDs ranged from 0.35 to 0.42 (all $P < 0.001$) following removal of each study one at a time from the meta-analysis (Figure 4).

Additional analyses

Subgroup analyses (SA) (Table 5) and meta-regression

Table 2 Study characteristics

Study	Country	Study design	*n* (active: control)	Transit time outcome, method	Probiotic strain	Daily dosage (10^9 CFU)	Delivery method	Treatment duration (d)
Agrawal *et al*[14], 2009	United Kingdom	Parallel groups	17:17	CTT, radiopaque markers	*B. lactis* DN-173 010	25	Active: Yogurt + probiotic Control: Nonfermented milk-based product	28
Bartram *et al*[15], 1994	Germany	Cross-over	12	OATT, radiopaque markers	*B. longum*	> 0.5	Active: Yogurt with 2.5 g lactulose + probiotic Control: Yogurt	21
Bazzocchi *et al*[25], 2014	Italy	Parallel groups	19:12	TITT, radiopaque markers	*L. plantarum*, *L. acidophilus*, *L. rhamnosus*, *B. longum*, *B. breve*	-	Active: Sachet with psyllium+probiotic Control: Sachet with 2.8 g maltodextrin	56
Bouvier *et al*[16], 2001	France	Parallel groups	36:36	CTT, radiopaque markers	*B. lactis* DN-173 010	97.5	Active: Probiotic fermented milk Control: Heat-treated probiotic fermented milk	11
Holma *et al*[17], 2010	Finland	Parallel groups	12:10	TITT, radiopaque markers	*L. rhamnosus* GG	20	Active: Buttermilk + probiotic and white wheat bread Control: White wheat bread	21
Hongisto *et al*[18], 2006	Finland	Parallel groups	16:14	TITT, radiopaque markers	*L. rhamnosus* GG	15	Active: Yogurt + probiotic and low fiber toast Control: Low fiber toast	21
Krammer *et al*[24], 2011	Germany	Parallel groups	12:12	CTT, radiopaque markers	*L. casei* Shirota	6.5	Active: Probiotic fermented milk drink Control: Nonfermented milk drink	28
Magro *et al*[26], 2014	Brazil	Parallel groups	26:21	CTT, radiopaque markers	*L. acidophilus* NCFM, *B. lactis* HN019	2	Active: Yogurt + polydextrose + probiotic Control: Yogurt	14
Malpeli *et al*[19], 2012	Argentina	Cross-over	83	OCTT, carmine red dye	*B. lactis* BB12	2-20	Active: Yogurt with 0.625 g inulin and oligofructose + probiotic	15
					L. casei CRL 431	2-12	Control: Yogurt	
Marteau *et al*[20], 2002	France	Cross-over	32	CTT, radiopaque markers	*B. lactis* DN-173 010	18.75	Active: Yogurt + probiotic Control: Yogurt	10
Merenstein *et al*[27], 2014	United States	Crossover	68	CTT, radiopaque markers	*B. animalis ssp. lactis Bf-6*	20-56	Active: Yogurt + probiotic Control: Yogurt	14
Rosenfeldt *et al*[21], 2003a	Denmark	Cross-over	13	GTT, radiopaque markers	*L. rhamnosus* 19070-2	20	Active: Freeze-dried powder + probiotic	18
					L. reuteri DSM 12246	20	Control: Skimmed milk powder w/dextrose	
Rosenfeldt *et al*[21], 2003b	Denmark	Cross-over	13	GTT, radiopaque markers	*L. casei* subsp. alactus CHCC 3137	20	Active: Freeze-dried powder + probiotic	18
					L. delbrueckii subsp. lactis CHCC 2329	20	Control: Skimmed milk powder w/dextrose	
					L. rhamnosus GG	20		
Sairanen *et al*[22], 2007	Finland	Parallel groups	22:20	CTT, radiopaque markers	*B. longum* BB536, *B. lactis* 420	2.4-18[1]	Active: Probiotic fermented milk	21
					L. acidophilus 145	0.48	Control: Fermented milk	
Tulk *et al*[28], 2013	Canada	Crossover	65	GTT, carmine red/carbon black capsules	*B. lactis* Bb12, *L. acidophilus* La5, *L. casei* CRL431	2	Active: Yogurt + probiotic + inulin Control: Yogurt	15
Waller *et al*[23], 2011a	United States	Parallel groups	33:34	WGTT; radiopaque markers	*B. lactis* HN019	1.8	Active: Capsule, maltodextrin, probiotic Control: Capsule, maltodextrin	14
Waller *et al*[23], 2011b	United States	Parallel groups	33:34	WGTT; radiopaque markers	*B. lactis* HN019	17.2	Active: Capsule, maltodextrin, probiotic Control: Capsule, maltodextrin	14

[1]Represents the reported range of total Bifidobacterium. CFU: Colony-forming units; CTT: Colonic transit time; GTT: Gastrointestinal transit time; OATT: Oro-anal transit time; OCTT: Oro-cecal TT; TITT: Total intestinal transit time; WGTT: Whole gut transit time.

Table 3 Subject characteristics

Study	Mean age (yr)	Female gender (%)	Mean BMI (kg/m^2)	Condition
Agrawal *et al*[14], 2009	40	100	25	IBS-C
Bartram *et al*[15], 1994	23	58	-[2]	None
Bazzocchi *et al*[25], 2014	40	86	19	Constipation
Bouvier *et al*[16], 2001	33	50	22	None
Holma *et al*[17], 2010	44	92[1]	24	Constipation
Hongisto *et al*[18], 2006	43	100	24	Constipation
Krammer *et al*[24], 2011	50	100	-[2]	Constipation
Magro *et al*[26], 2014	32	91	28	Constipation
Malpeli *et al*[19], 2012	41	100	-[2]	Constipation
Marteau *et al*[20], 2002	27	100	21	None
Merenstein *et al*[27], 2014	29	100	23	None
Rosenfeldt *et al*[21], 2003a	25	0	-[2]	None
Rosenfeldt *et al*[21], 2003b	25	0	-[2]	None
Sairanen *et al*[22], 2007	39	64	25	None
Tulk *et al*[28], 2013	29	60	24	None
Waller *et al*[23], 2011a	44	65	31	Constipation
Waller *et al*[23], 2011b	44	65	32	Constipation

[1]Percentage estimated from larger study cohort; [2]Represents missing data. BMI: Body mass index; IBS-C: Irritable bowel syndrome, constipation predominant.

Table 4 Assessment of study quality

Study	Jadad scale			
	Randomization range: 0-2	Double blinding range: 0-2	Subject account range: 0-1	Total score[1] range: 0-5
Agrawal *et al*[14], 2009	1	2	1	4
Bartram *et al*[15], 1994	1	2	0	3
Bazzocchi *et al*[25], 2014	1	2	1	4
Bouvier *et al*[16], 2001	1	2	0	3
Holma *et al*[17], 2010	1	0	1	2
Hongisto *et al*[18], 2006	1	0	0	1
Krammer *et al*[24], 2011	1	1	1	3
Magro *et al*[26], 2014	2	2	1	5
Malpeli *et al*[19], 2012	0	2	1	3
Marteau *et al*[20], 2002	1	2	1	4
Merenstein *et al*[27], 2014	2	2	1	5
Rosenfeldt *et al*[21], 2003a	1	1	0	2
Rosenfeldt *et al*[21], 2003b	1	1	0	2
Sairanen *et al*[22], 2007	1	1	0	2
Tulk *et al*[28], 2013	1	1	1	3
Waller *et al*[23], 2011a	2	2	1	5
Waller *et al*[23], 2011b	2	2	1	5

[1]Higher scores represent better study quality.

Table 5 Subgroup analysis of study- and subject-related factors on intestinal transit time

Study	SMD	95%CI	*P* value (pre-post)	*P* value (between groups)
Subject condition				
Constipation/IBS-C (n = 9)	0.57	0.39-0.75	< 0.001	< 0.01
Healthy (n = 8)	0.22	0.05-0.39	0.01	
Study quality				
Jadad score ⩾ 3 (n = 12)	0.45	0.31-0.59	< 0.001	0.01
Jadad score < 3 (n = 5)	0.00	-0.33-0.33	> 0.99	
Age[1]				
⩾ 39 yr (n = 9)	0.51	0.29-0.73	< 0.001	0.08
< 39 yr (n = 8)	0.27	0.09-0.44	< 0.01	
Publication year				
After 2008 (n = 10)	0.47	0.29-0.65	< 0.001	0.08
Before 2008 (n = 7)	0.20	-0.03-0.44	0.09	
Number of probiotic strains				
Single strain (n = 10)	0.49	0.32-0.66	< 0.001	0.09
Multiple strains (n = 7)	0.23	-0.01-0.47	0.06	
Study design				
Parallel groups (n = 11)	0.48	0.31-0.65	< 0.001	0.09
Cross-over (n = 6)	0.26	-0.02-0.46	0.07	
Body mass index[1,2]				
⩾ 25 kg/m^2 (n = 5)	0.59	0.24-0.94	< 0.001	0.16
< 25 kg/m^2 (n = 7)	0.31	0.13-0.49	< 0.001	
Treatment duration[1]				
< 18 d (n = 8)	0.45	0.29-0.60	< 0.001	0.17
⩾ 18 d (n = 9)	0.22	-0.06-0.50	0.12	
Geographic location				
Americas (n = 6)	0.47	0.26-0.67	< 0.001	0.20
Europe (n = 11)	0.28	0.07-0.49	< 0.01	
Female gender proportion[1]				
⩾ 86% (n = 9)	0.47	0.30-0.64	< 0.01	0.22
< 86% (n = 8)	0.27	0.00-0.54	< 0.05	
Confounding treatments[3]				
Yes (n = 7)	0.46	0.24-0.67	< 0.001	0.32
No (n = 10)	0.30	0.10-0.51	< 0.01	
Daily probiotic dosage[1]				
⩾ 1.6^{10} CFU (n = 8)	0.40	0.12-0.67	< 0.01	0.74
< 1.6^{10} CFU (n = 7)	0.34	0.16-0.52	< 0.001	

[1]Categorized by median value; [2]Body mass index not reported for 5 treatment effects; [3]Includes studies where treatment included probiotics plus fiber or non-digestible sugar. Variables sorted from lowest to highest between-groups *P* value; n represents the number of treatment effects. IBS-C: Irritable bowel syndrome, constipation predominant; SMD: Standardized mean difference.

(MR) (Table 6) were performed to determine the influence of study- and subject-related characteristics on ITT. Probiotic supplementation reduced ITT in comparison to controls in several of the analyzed subgroups. Greater reductions in ITT were observed with probiotics in subjects with *vs* without constipation (SA and MR, $P < 0.01$) and in high-quality (Jadad score ⩾ 3) *vs* low-quality (Jadad score < 3) studies (SA and MR, $P = 0.01$). There were trends for greater probiotic efficacy with older age (SA, $P = 0.08$, MR, $P = 0.02$), in recently published studies (SA, $P = 0.08$), with parallel groups study designs (SA, $P = 0.08$), higher percentage of female subjects (SA, $P = 0.08$,

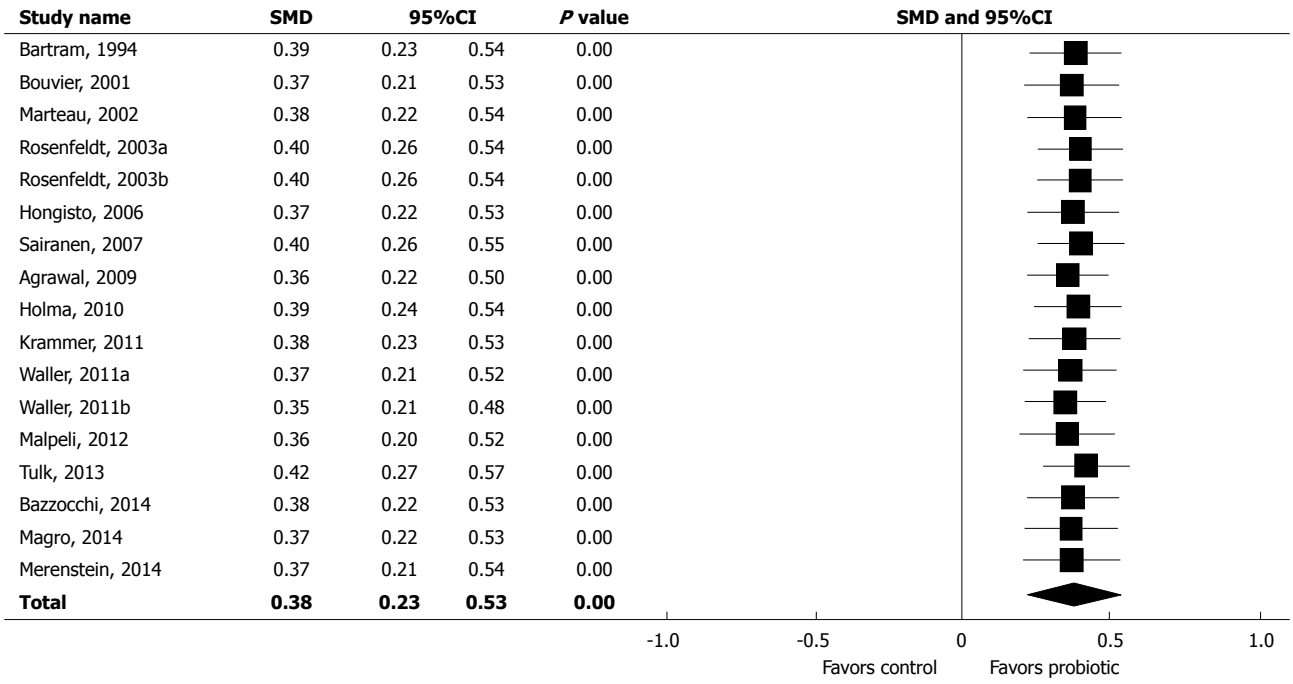

Figure 4 One study removed forest plot of standardized mean difference in intestinal transit time across studies. SMD: Standardized mean difference.

Table 6 Meta-regression of study- and subject-related factors on intestinal transit time

Variable	Unit of measure	Intercept	Point estimate	Explained variance (%)	*P* value
Constipation/ IBS-C	1 = Yes; 0 = No	0.218	0.352	38	< 0.01
Jadad score	Per 1 unit	-0.117	0.141	31	0.01
Age	Per 1 yr	-0.352	0.021	27	0.02
Female gender proportion	Per 10%	-0.045	0.055	26	0.02
Number of probiotic strains	Per 1 strain	0.618	-0.133	20	< 0.05
Body mass index[1]	Per 1 kg/m^2	-0.526	0.037	22	0.08
Treatment duration	Per 1 d	0.392	-0.004	0	0.96
Daily probiotic dosage	Per 10 × 10^9 CFU	0.385	-0.001	0	0.98

[1]Body mass index not reported for 5 treatment effects. Variables sorted from greatest to least explained variance.

Table 7 Subgroup analysis of probiotic strains on intestinal transit time

Probiotic strain	No. of treatment effects	SMD	95%CI	*P* value
B. lactis HN019	3	0.67	0.37-0.97	< 0.001
B. lactis DN-173 010	3	0.54	0.16-0.92	< 0.01
L. casei CRL 431	2	0.33	-0.10-0.75	0.14
B. lactis BB12	2	0.33	-0.10-0.75	0.14
L. rhamnosus GG	3	0.10	-0.35-0.55	0.67

Probiotic strains sorted from highest to lowest standard mean difference. SMD: Standardized mean difference.

MR, $P = 0.02$), single-strain probiotics (SA, $P = 0.09$, MR, $P < 0.05$) and higher body mass index (SA, $P = 0.16$, MR, $P = 0.08$). Treatment duration, geographic location of study, inclusion of potentially confounding treatments, and daily probiotic dosage were not found to have a significant influence on probiotic efficacy in subgroup analysis and meta-regression. Analysis of outcomes by probiotic strain identified medium to large treatment effects with *B. lactis* HN019 (SMD: 0.67, $P < 0.001$) and *B. lactis* DN-173 010 (SMD: 0.54, $P < 0.01$) while treatment effects with other strains were small (SMD: 0.10-0.33) and not statistically significant (Table 7).

DISCUSSION

An ever-increasing body of evidence implicates the gastrointestinal microbiome in defining states of health and disease[29]. Probiotics may restore the composition of the gut microbiome and support beneficial functions to gut microbial communities, resulting in amelioration of gut inflammation and other disease phenotypes[30]. Consequently, probiotic supplementation is increasingly touted as an effective and accessible means of improving gut health, even in the general population of healthy adults. The current systematic review and meta-analysis demonstrates that short-term probiotic supplementation yielded moderate ITT reductions in adults. Additionally, the treatment effect of probiotics was greater in subjects with constipation, in high-quality studies, and with certain probiotic strains. In contrast to the moderate treatment effect observed in constipated subjects, probiotics only minimally influenced ITT in non-constipated adults. Given this finding, it appears that probiotic consumption will

not lead to undesired short ITT or diarrhea. However, probiotic consumption for the sole purpose of reducing ITT is unjustified in healthy adults. Nevertheless, this finding does not diminish other beneficial effects that have been observed with probiotics in healthy adults[31,32].

In this meta-analysis, there was a trend for greater treatment effects with probiotics in parallel groups study designs compared to crossover studies (SMD: 0.48 *vs* 0.26, $P = 0.09$). Although there is no clear explanation for this finding, data from one included study deserves further discussion. The study of Merenstein *et al*[27] enrolled 68 healthy women using a crossover design, with a 6-wk washout between treatment periods. However, a significant carry-over effect was observed at the start of the second treatment period. For purposes of this meta-analysis, we treated this study as a parallel groups design using data from the first treatment period only[33]. Although the presence of a carry-over effect was not mentioned in the other crossover studies included in this analysis, the fact that washout periods ranged from 2 to 6 wk with significant carryover identified even after 6 wk in the Merenstein study raises the question of whether carry-over effects may have influenced outcomes of other crossover studies. Although crossover studies may initially appear attractive to researchers given the smaller sample size requirements compared to parallel groups designs, we propose that crossover designs are inappropriate in probiotic clinical trials unless the washout period for the probiotic has been previously established for the specific condition under study.

In comparison to our previous meta-analysis on this topic, the treatment effect of probiotics on ITT was largely unchanged (SMD: 0.40 *vs* 0.38). Importantly, with the addition of more studies, we were able to explore potential sources of heterogeneity among studies with greater precision. Novel subgroup findings included the observation of moderate probiotic treatment effects (SMD: 0.45) in high-quality studies, but no treatment effect (SMD: 0.0) in low-quality studies. Although the treatment effect sizes in parallel groups and crossover studies remained largely unchanged, study design is now a considerably stronger predictor of heterogeneity in ITT outcomes given the inclusion of additional studies. We also identified that single-strain probiotics were more efficacious than multiple strain probiotics. Although *B. lactis* HN019 and *B. lactis* DN-173 010 remained the most efficacious probiotic strains, we were able to analyze additional probiotic strains that yielded modest improvements in ITT relative to placebo.

The strengths of this systematic review and meta-analysis are inclusion of only RCTs and a comprehensive assessment of the influence of moderator variables on ITT with probiotic supplementation. Our study also revealed several limitations in the design of ITT studies with probiotics. First, the treatment duration of included studies ranged from 10 to 56 d. Although the long-term safety of probiotics is well established[34], probiotic efficacy on ITT beyond 8 wk cannot be interpreted with the current analysis. Second, although the therapeutic benefit of probiotics appears to be strain-specific, the small number of studies performed with each strain prevented robust strain-specific comparisons. Finally, subject characteristics were relatively homogenous among studies with regard to age and gender. Therefore, the generalizability of these findings to the general population, particularly males and the elderly, is unknown. These findings give specific suggestions for future research in this field.

In conclusion, probiotic supplementation is moderately efficacious for reducing ITT in adults. Probiotics were most efficacious in constipated subjects, when evaluated in high-quality studies, and with certain probiotic strains.

COMMENTS

Background

Functional gastrointestinal disorders are common in the general population, with slow intestinal transit a common symptom. No therapy is highly efficacious, safe, and cost effective for treatment of slow-transit bowel disorders. Probiotics have been extensively studied for treatment of gastrointestinal disorders and may confer improvements in bowel regularity.

Research frontiers

Clinical trials of probiotic supplementation on intestinal transit time (ITT) yield discrepant results. The authors performed a contemporary systematic review and meta-analysis on the efficacy of probiotic supplementation on ITT in adults, with a secondary focus on exploring sources of heterogeneity through meta-regression and subgroup analyses.

Innovations and breakthroughs

Probiotics are most efficacious in constipated subjects, when evaluated in high-quality studies, and with certain probiotic strains.

Applications

Probiotic supplementation appears to confer clinically meaningful improvements in intestinal transit in subjects with constipation. Probiotic efficacy also significantly differs according to strain.

Terminology

Probiotics are live micro-organisms that confer a health benefit on the host when administered in adequate dosages. Intestinal transit time is an indicator of the time taken for a food bolus to travel through the gastrointestinal system. The standardized mean difference is a statistical measure of effect size for continuous outcomes, defined as the mean difference between groups divided by the pooled standard deviation.

Peer-review

Very nice manuscript.

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CASE REPORT

Modified single transluminal gateway transcystic multiple drainage technique for a huge infected walled-off pancreatic necrosis: A case report

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Kosuke Minaga, Masayuki Kitano, Hajime Imai, Kentaro Yamao, Ken Kamata, Takeshi Miyata, Tomohiko Matsuda, Shunsuke Omoto, Kumpei Kadosaka, Tomoe Yoshikawa, Masatoshi Kudo, Department of Gastroenterology and Hepatology, Kinki University Faculty of Medicine, Osaka-Sayama 589-8511, Japan

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Abstract

We report a successful endoscopic ultrasonography-guided drainage of a huge infected multilocular walled-off necrosis (WON) that was treated by a modified single transluminal gateway transcystic multiple drainage (SGTMD) technique. After placing a wide-caliber fully covered metal stent, follow-up computed tomography revealed an undrained subcavity of WON. A large fistula that was created by the wide-caliber metal stent enabled the insertion of a forward-viewing upper endoscope directly into the main cavity, and the narrow connection route within the main cavity to the subcavity was identified with a direct view, leading to the successful drainage of the subcavity. This modified SGTMD technique appears to be useful for seeking connection routes between subcavities of WON in some cases.

Key words: Endoscopic ultrasonography; Infected pancreatic necrosis; Walled-off necrosis; Endoscopic ultrasonography-guided drainage; Acute pancreatitis

Core tip: Walled-off necrosis (WON) remains difficult to endoscopically manage because of insufficient drainage of solid necrotic tissues. Here, we present a case of successful drainage of a huge WON *via* a modified single transluminal gateway transcystic multiple drainage technique. After placing a wide-caliber covered metal stent, follow-up computed tomography revealed an undrained subcavity of WON. A large fistula created by the metal stent enabled the insertion of an upper endoscope directly into the main cavity, and the narrow connection route within the main cavity to the subcavity was identified with a direct view, leading to the successful drainage of the subcavity.

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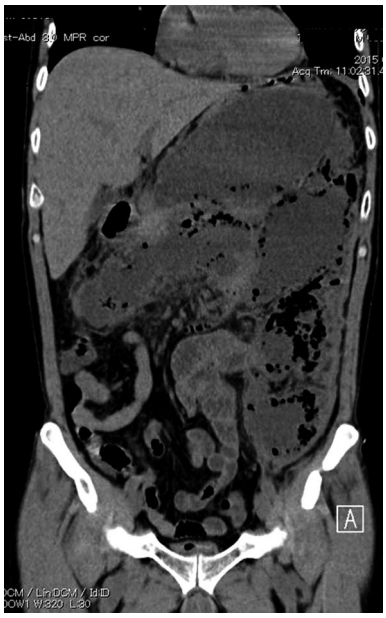

Figure 1 Abdominal computed tomography scan showing a huge multilocular walled-off necrosis replacing the body and tail of the pancreas, which extended to the pelvis. Gas bubbles were observed in the cavity.

INTRODUCTION

Endoscopic ultrasonography (EUS)-guided drainage for pancreatic fluid collection (PFC) is increasingly used as a minimally invasive alternative to surgical and percutaneous drainage[1-3]. However, walled-off necrosis (WON) remains difficult to endoscopically manage because of insufficient drainage of solid necrotic tissues. Various techniques, such as the use of wide-caliber metal stents[4,5], direct endoscopic necrosectomy[6,7] and multiple transluminal gateway technique[8] are reportedly useful for managing WON. However, responses to these advanced techniques remain unsatisfactory in some cases. Recently, a single transluminal gateway transcystic multiple drainage (SGTMD) was developed for treating complicated multilocular WON[9]. Here, we present a case of successful endoscopic drainage of a huge infected multilocular WON *via* a modified SGTMD technique.

CASE REPORT

A 49-year-old male presented with upper abdominal pain and high fever of 7 d duration. He was diagnosed with alcohol-induced severe acute pancreatitis 1 mo before and was discharged 6 d after admission from a neighbouring general hospital. His computed tomography (CT) severity index[10] was 6. He was re-admitted to our hospital with the above-mentioned chief complaints. Laboratory tests revealed elevated C-reactive protein (CRP) and procalcitonin levels (27.8 mg/dL and 6.17 ng/mL, respectively). Elevated levels of kidney function parameters were also noted (blood urea nitrogen level, 77 mg/dL; serum creatinine level, 3.14 mg/dL). An abdominal CT revealed a huge multilocular WON measuring 31 cm × 16 cm, which spread from the pancreas to pelvis (Figure 1). Clinically, infection of the necrosis was assumed. Doripenem was intravenously introduced; however, his clinical symptoms and elevated inflammatory reaction persisted. As the main cavity of WON was close to the gastric lumen, we decided to puncture WON under EUS guidance. EUS-guided transluminal drainage was performed; a wide-caliber fully covered TTS Niti-S esophageal stent (internal diameter, 16 mm; maximum flange diameter, 24 mm; length, 40 mm; Taewoong Medical, Seoul, South Korea) was placed (Figure 2). Through the metal stent, a 7-Fr double-pigtail plastic stent (length, 80 mm) and a 7-Fr nasocystic catheter were inserted (Figure 3). During the procedure, approximately 2.4 L of purulent fluid were suctioned. A follow-up abdominal CT obtained 1 wk after the procedure demonstrated a significant reduction in the size of the main cavity; however, the undrained subcavity remained, which was mainly located in the left anterior pararenal space and extended to the left pelvis (Figure 4). Additional drainage targeting the subcavity was required because high fever continued after the procedure. Because the subcavity was not adjacent to the stomach or duodenum, additional EUS-guided puncture was difficult. CT suggested communication between the subcavity and main cavity; therefore, a SGTMD procedure was considered. Repeated attempts to determine the connection route within the main cavity to the subcavity using an ERCP catheter and 0.025-inch guidewire were unsuccessful. The metal stent was removed, and a large fistula that was created by the metal stent enabled the insertion

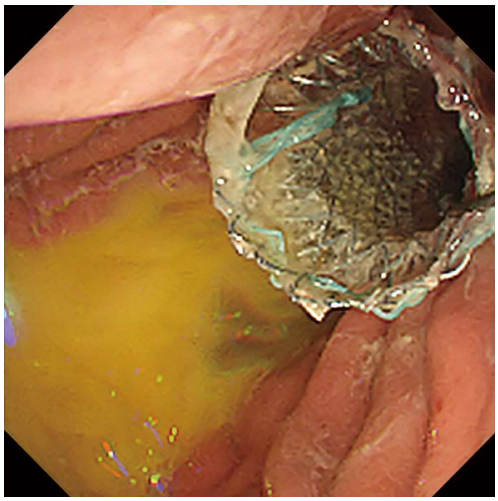

Figure 2 Successful deployment of a wide-caliber fully covered TTS Niti-S esophageal stent. Purulent fluid was observed in the gastric lumen.

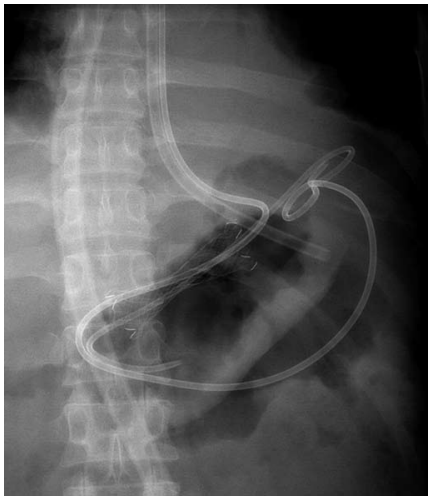

Figure 3 A 7-Fr double-pigtail plastic stent and a 7-Fr nasocystic catheter were deployed through the fully covered metal stent.

of a forward-viewing upper endoscope directly into the main cavity. After the endoscope was advanced into the cavity, a narrow connection route was identified (Figure 5). Contrast medium was injected into the connection. Having confirmed the detection of the subcavity, the guidewire was inserted into the cavity and two 7-Fr double-pigtail plastic stents (lengths, 120 and 80 mm, respectively) were deployed (Figure 6). No procedure-related complications were observed. After additional endoscopic management, high fever resolved over the course of a few days and CRP levels significantly decreased. CT revealed that the subcavity of WON was well drained. The patient completely recovered and was discharged after 3 wk of hospitalization. Follow-up CT obtained 1 month after discharge revealed that WON had mostly collapsed (Figure 7) and the patient remained symptom free.

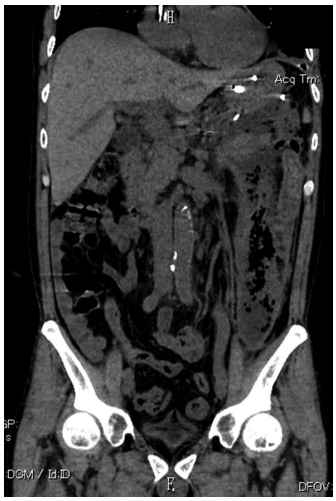

Figure 4 Computed tomography one week after initial drainage showed an undrained subcavity, located mainly at the left anterior pararenal space that extended to the left pelvis.

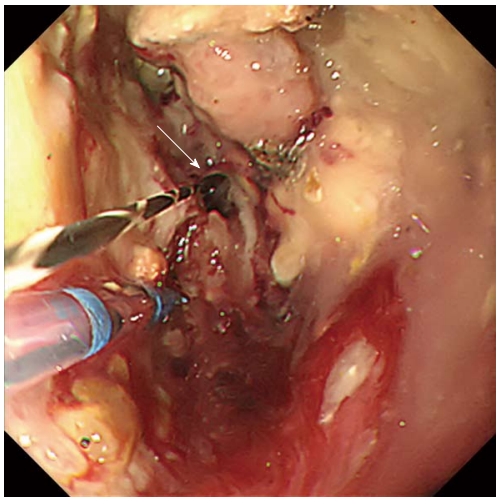

Figure 5 Endoscopic view of the cavity of walled-off necrosis by a modified single transluminal gateway transcystic multiple drainage technique. An upper endoscope was inserted into the walled-off necrosis (WON) through the fistula and a narrow connection route within the main cavity to the subcavity could be identified directly (white arrow).

DISCUSSION

Over the last decade, techniques for pancreatic fluid collection have shifted toward minimally invasive approaches. Since first reported in 1992 by Grimm *et al*[1] EUS-guided transluminal drainage for pancreatic fluid collection has played a pivotal role and spread worldwide as a minimally invasive alternative to surgical and percutaneous drainage[1-3]. However, the clinical response rate of the conventional single transluminal gateway technique deploying single or multiple stenting for treating WON is not satisfactory (described as 45%-63%)[8,11]. Recently, various techniques, such as the use of wide-caliber metal

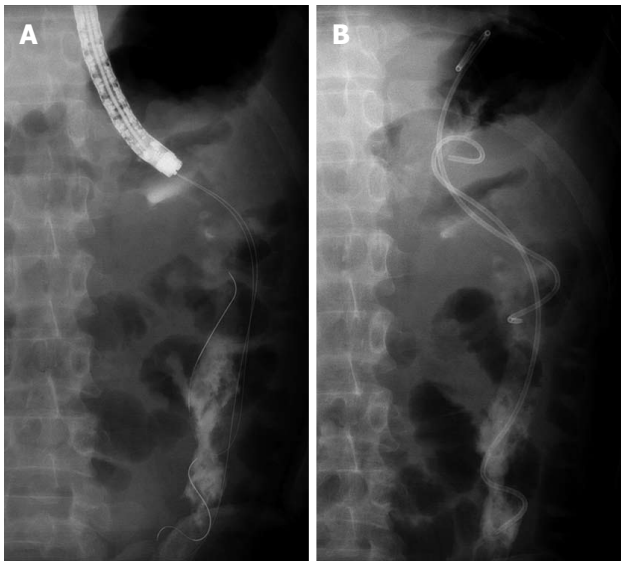

Figure 6 Fluoroscopic view of modified single transluminal gateway transcystic multiple drainage technique. With a direct view of the connection route, a 0.025-inch guidewire was inserted into the subcavity (A) and two 7-Fr double-pigtail plastic stents were deployed (B).

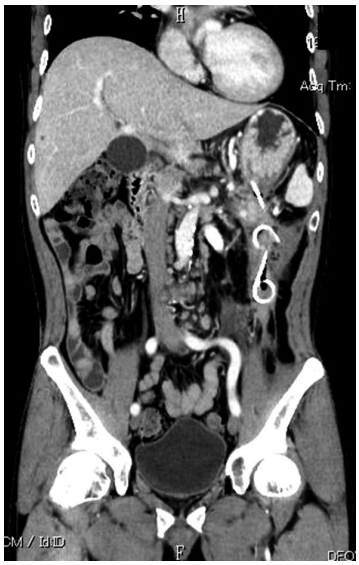

Figure 7 Follow-up computed tomography obtained one month after discharge revealed the WON had mostly collapsed.

stents[4,5], direct endoscopic necrosectomy[6,7] and multiple transluminal gateway technique[8] have improved the clinical success rate of endoscopic management of WON. However, response to these advanced techniques remains unsatisfactory in some cases. Mukai *et al*[9] recently described a novel SGTMD procedure for complicated multilocular WON and reported successful drainage in five cases using this technique. When subcavities are located far from the gastrointestinal lumen, percutaneous approach would have been used conventionally. Mukai *et al*[9] hypothesized that the multilocular cavity may have originally been unilocular and separated into subcavities with tiny, narrow connections during the process of treatment and collapse. They used an ERCP catheter and soft guidewire to locate tiny, narrow connections. In this case, we repeatedly attempted to identify the connection using an ERCP catheter and soft guidewire through the metal stent under fluoroscopic guidance, but the guidewire curled up in the main cavity and failed to locate a connection route. Instead, we inserted the upper endoscope into the cavity through the large fistula, which enabled the narrow connection route to be directly observed. The guidewire was easily and safely advanced into the subcavity, and successful drainage of the subcavity was achieved. This is a modified technique of the previously described SGTMD. In addition to SGTMD, having a direct view to identify the connection route may lead to a higher success rate in some cases.

In this case, the pig-tail stents have been left in place during 6 mo follow-up. This is because the previous studies revealed that stent retrieval was associated with higher PFC recurrence rates[12,13].

In conclusion, we presented a case of successful endoscopic drainage of a huge infected multilocular WON by a modified SGTMD technique with direct endoscope insertion into the cavity. This modified SGTMD technique appears to be useful in seeking connection routes between the subcavities of WON and might avoid the requirement for a more invasive drainage procedure, such as endoscopic or surgical necrosectomy.

COMMENTS

Case characteristics

One month after being diagnosed with alcohol-induced severe acute pancreatitis, a 49-year-old male presented with upper abdominal pain and high fever of 7 d duration.

Clinical diagnosis

The patient had upper abdominal pain and high fever.

Differential diagnosis

Pancreatic pseudocyst.

Laboratory diagnosis

The laboratory findings showed elevated C-reactive protein, procalcitonin levels and renal dysfunction.

Imaging diagnosis

Abdominal computed tomography demonstrated a huge multilocular WON measuring 31 cm × 16 cm, which spread from the pancreas to pelvis.

Pathological diagnosis

Pathological examination was not performed in this case.

Treatment

Endoscopic drainage with a modified single transluminal gateway transcystic multiple drainage (SGTMD) technique was performed.

Related reports

WON remains difficult to endoscopically manage because of insufficient drainage of solid necrotic tissues. Various techniques, such as the use of wide-caliber metal stents, direct endoscopic necrosectomy, multiple transluminal gateway technique and SGTMD technique were developed for treating WON.

Term explanation

Modified SGTMD is a novel alternative technique for drainage of WON which means a single transluminal gateway transcystic multiple drainage with direct endoscope insertion into the cavity.

Experiences and lessons

Modified SGTMD technique appears to be useful in seeking connection routes between the subcavities of WON and might avoid the requirement for a more invasive drainage procedure, such as endoscopic or surgical necrosectomy.

Peer-review

This case report is interesting and well documented.

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EDITORIAL

Need of righteous attitudes towards eradication of hepatitis C virus infection in Latin America

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Abstract

Over the last few years, we have expanded our knowledge on numerous facets of the hepatitis C virus (HCV). Beginning with its discovery and viral life cycle, its impact on health, the development of liver disease and currently, effective antiviral treatments. The latter point has become of great interest throughout the developed world, where the possible eradication of HCV through specific strategies to reach all HCV-infected people has been announced. However, this scenario is very different in the countries of Latin America (LA), in which < 2% of infected patients requiring treatment have access to HCV medications. It has been estimated that at least ten million Latin Americans may be infected with HCV. Despite the numbers, viral hepatitis does not seem to be considered a health problem in this region of the world. This reality poses a challenge for politicians and governments of these countries, as well as to the pharmaceutical industry, the medical practitioners, and academics in LA. In this editorial, we state the need for alterations in the attitudes of the integral players involved in this situation. A recognition shift could help to create preventive strategies of viral hepatitis and to advocate for accessibility to new HCV treatments.

Key words: Low-income; Antiviral agents; Public health; Medical societies; Drug industry

Core tip: Global eradication of hepatitis C virus (HCV) infection is causing considerable interest, especially in the developed world. However, the accessibility to the new direct-acting antiviral regimens in low- and middle- income countries is an unmet need. At least ten million HCV-infected persons in Latin America (LA) are confronted by multiple barriers to HCV treatment. Moreover, for the LA countries, paradoxically at it seems, money may not be the only issue. The health authorities, the medical community, and the pharmaceutical industry are the key players that need to alter their attitude towards the delivery of HCV treatments to all patients irrespective of their socio-

economic status.

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INTRODUCTION

Early years of hepatitis C virus infection

The history of the hepatitis C virus (HCV) is a strong example of a bench-to-bedside approach illustrating how basic scientific knowledge is applied to patient care.

Following the discovery of hepatitis B virus (HBV) in 1965[1], and hepatitis A virus (HAV) in 1973[2], a clinical entity known as post-transfusion non-A non-B (NANB) hepatitis became more evident[3]. This was regardless of the banning of commercial blood donations systems, and the implementation of HAV and HBV blood testing in 1975[3,4]. Furthermore in 1989, after years of intensive research[5], a scientific breakthrough discovered another virus consisting of a single positive strand of RNA, the HCV, classified in the *Flaviridae* family[6]. HCV was determined to be the causing agent of the post-transfusion NANB hepatitis[4,5].

In 1992, following the cloning of HCV, an evolving series of sensitive and specific anti-HCV antibody immunoassays were developed which allowed for the mandatory testing of blood collections in the United States[3]. Subsequently, third-generation immunoassays and RNA-HCV nucleic acid amplification tests improved the diagnostics of HCV for blood transfusion safety[7]. During the 90's, the HCV genotypes and subtypes, as well as their heterogeneous geographic distribution became apparent through basic research and molecular epidemiology[8]. Additionally in the United States, blood transfusions, injection drug use (IDU) and several medical procedures were identified as high-risk factors[3].

During the 90's but before 2011, the first antivirals to be marketed were standard interferon, followed by pegylated-interferon which was later combined with ribavirin[9]. Unfortunately, these antivirals achieved a low-level of sustained viral response (SVR) among patients with HCV genotype 1 compared to other genotypes[10]. However after 2011, the first and second generation direct-acting antivirals based on targeting the specific molecules of the HCV life cycle have proven more efficient regardless of genotype reaching an SVR in more than 90% or 95% of the patients who receive treatment[10].

In recent years, DNA sequencing and bioinformatics of HCV strains worldwide have allowed the evolutionary analysis and molecular tracing of the HCV epidemic across countries, mainly focusing on genotype 1a and 1b[11]. These studies have documented the prevalence of HCV in Japan since 1920, in Europe since 1940 and the United States since 1960[11,12]. Further studies have confirmed that the introduction of HCV in the United States may have occurred even earlier (in the 1950's) and is related to the use of contaminated blood obtained from commercial blood donors and the return of World War Ⅱ soldiers[13]. These reasons, along with the fact that hepatitis C is a stealthy virus causing injury to the liver in a silent manner, people who were born from 1945-1965 are currently in the target population of the "baby boomers" screening campaign for HCV in the United States[14].

Transmission of HCV infection in Latin America

Mexico's geographic location serves as a common pathway of intracontinental migration between the United States and other countries of LA. Thus, HCV could have spread from the United States to this region. However in LA, awareness of an NANB hepatitis did not intensify until the late 1970s and early 1980s[15]. During this period, the United States veterans returning from Vietnam, as well as the migration of Latin Americans to their homeland as retirees or by multiple processes of deportation and migration between countries became a major social-demographic phenomenon.

The first HCV epidemic outbreak was associated with the emergence of HIV in the mid-1980s, at a time where the presence of paid blood donors was a common situation[16]. This parenteral route of transmission may have been the primary source of dispersion in Central and South America since IDU, during those decades, was very rare in LA[17]. Furthermore, even though in the United States mandatory screening of blood-borne viruses had been implemented in 1992, it was not fully established in Mexico and LA until 1996[18,19]. This information indicates that even before 2000, blood donations contaminated with HCV were still a relevant risk factor[20]. Moreover, an additional route of HCV infection in the LA may have been the indiscriminate use of caesarean births and other medical procedures that took place during the same period[21]. Brazil and Mexico[22] are the two countries with the highest rates of cesarean sections within the Americas[23]. This could explain why Mexico has a female-to-male ratio of 2:1 of HCV infection[21].

Although partial reports were stating the frequency of HCV in countries of LA during the 1990s, it was not until after 2000 that HCV-related liver cirrhosis began to rise. In Mexico, this disease entity represents over 12 deaths per 100000 inhabitants and the estimated number of people infected with HCV may range from 1.5 to 2 million[24-26], and at least ten million in LA[26-28]. In this region the route of transmission of HCV may have been similar as it was in Mexico, taking into account that as of 2000 the use of IDU, as well as tattooing and piercing, had increased from that year

forward[17].

HCV TREATMENT IN LATIN AMERICA

HCV infection can only be prevented by evading or eradicating the virus. To achieve eradication, treatment with highly effective drugs to all who are infected is required. Currently with the introduction of the new, but very costly, direct-acting antivirals (DAAs) an enormous feeling of success is felt. This elation may be comprehensible for the developed countries, but it may not be true for many developing countries worldwide[29-32], including those in LA[33-37].

However, there are two sides to this story. On one hand, the feasibility of eradicating HCV is closer than ever due to new treatments[38]; yet, on the other hand, neither the authorities nor the physicians, recognize that many infected patients are the result of an iatrogenic spread of HCV. More than 95% of patients infected with viral hepatitis belong to a lower social class in their respective countries, and they can not afford to pay the current market prices of these new antiviral drugs[39].

Additionally, a significant challenge for the eradication of HCV is that there is more than one genotype of the human virus. There are seven genotypes and multiple subtypes[40], which have infected populations with heterogeneous genetic makeups worldwide. This point indicates that research data on HCV needs to be population-based, especially in LA. Further studies are required to identify the genotypes that circulate[41,42], the main risk factors[17,20,21] and the immunogenetic background of the population[43-45].

To date, less than 2% of the people infected with HCV have been treated with the standard pegylated-interferon/ribavirin therapy. The new DAAs, such as boceprevir, telaprevir, simeprevir, and sofosbuvir have been slowly licensed in a limited number of countries in LA. Moreover, HCV treatments are based on the United States and European guidelines which provide evidence of the SVR obtained in clinical trials carried out in populations other than LA.

Thus, under these circumstances who will have access to treatment in Latin America? A substantial body of literature has documented the multiple barriers to health care in HCV-infected patients[46-49]. Although crucial, they are not within the scope of this editorial. Alternatively, we address several issues regarding the health authorities, the medical community, and the pharmaceutical industry. These are key players involved in the prevention, diagnosis, and treatment of this disease.

KEY PLAYERS

Health authorities

The health officials have not considered viral hepatitis to be a severe health problem, which in turn has manifested ignorance or reluctance towards a state program of detection or treatment of this disease. Consequently, there are a limited number of up-to-date population-based epidemiological studies of viral hepatitis in most LA countries[35,36] sponsored by the government. The few that do exist are accomplished by the attention and personal interest of researchers, rather than the concern of the health authorities. This lack of concern from the health authorities translates into key factors and information being overlooked. Some of these factors include; lack of precision of who and how many people are infected, primary risk factors involved[50], transition in genotypes[17] and co-morbidities (obesity, diabetes, alcoholism, co-infection with HVB and HIV)[51]. Furthermore, these epidemiological studies need to be documented in the population of LA because most clinicians assume that the same SVR achieved in Caucasians will replicate in people from this region.

Medical community

In LA, including Mexico, most medical societies involved in the study of the liver have a limited number of members with a solid scientific career, based on their poor scientific productivity in indexed and high impact factor journals in their dedicated fields[52,53]. These society members are hosted by the pharmaceutical companies to attend international forums. In turn, they are the only speakers that echo their experience at the liver meetings to their fellows but do not contribute with scientific data or share their clinical experience. This circumstance has led to the situation that some members only intend to be their society's President (or Chairperson) without the actual contribution of new knowledge in their field of expertise. Moreover, it is precisely these "leaders of the field" who take part, on multiple occasions, as representatives of the health authority, sometimes playing a dual role as clinician and politician.

Therefore, when the support of the medical community is required to establish health policies that have an impact on vulnerable social groups, the members are left without a voice, and vote, on these issues. Consequently, the decisions are made in an unipersonal manner. Furthermore, if the clinician-politician now has a position within the health institutions or the government, he/she may now have a conflict of interest with his/her private practice.

Unfortunately, the lack of an official standpoint in many educational or health institutions to promote a high standard of scientific and academic quality is a serious weakness. This challenge, paired together with a high level of corruption and preferential treatment that prevails among some government authorities, makes it tough to provide treatment for this virus. This situation has created elitist groups of treating physicians being sponsored by the pharmaceutical industry. These doctors may be knowledgeable, however, in some cases, this information does not

necessarily apply to their respective countries or communities.

Furthermore, many of these physicians focus on his/her private practice and have negligible interest in understanding the reality of the disease in their community or country of origin. A reminder of the Hippocratic Oath is "We as doctors should seek the best benefit for the patients, including those who do not have access to treatment".

Likewise, in the majority of LA countries, there are no specialists in hepatology. Patients living with liver diseases are treated mainly by a gastroenterologist or internist[54]. However, with the introduction of the new effective antivirals, and the possibility of the pharmaceutical industry involvement, medical specialists from different fields now claim the liver-diseased patient. Thus, a specialty or subspecialty in hepatology supported by academic institutions should be established as soon as possible.

Pharmaceutical industry

The pharmaceutical industry has declared a responsibility to eradicate HCV by creating agreements based on the economy of each country for the marketing of the antiviral drugs. In general, the standard procedure for the pricing of these drugs is to achieve a full refund of the R and D investment in the developed countries, intermediate reimbursement in the middle-income countries, and the possibility to offer a generic product at a lower and accessible cost for the low-income countries (defined as a win-win situation).

Unfortunately, there are still some well-known challenges to overcome in many developing countries, such as corruption and slow bureaucracy to introduce these drugs[55]. Additionally, the lack of precise epidemiological information about HCV infection is an ongoing difficulty.

Another obstacle is performing the diagnostic tests. Each patient should be evaluated before treatment because not all patients that are positive for anti-HCV antibodies have detectable viral loads or have a similar grade of liver damage. Thus, a pretreatment algorithm including initial and follow-up viral loads, genotyping and identification of possible resistance mutations, and staging of liver damage (fibrosis/cirrhosis) should be considered. A conservative estimate of the costs of these tests may exceed up to $2000 to $4000 United States dollars before paying at least $10000 to $80000 United States dollars for a three-month period of treatment[56]. If the patient is a potential non-responder or present with advanced liver damage, this cost may rise even higher.

Considering these conditions several options have been proposed, such as the establishment of non-profit societies that aid in the funding of these high costs for the poor. This creates a risky situation, given the lack of transparency and poor attitudes of some key players previously mentioned, there is a chance that these "non-profit" organizations may end up as personal or family businesses, or in the hand of small groups who are in power.

Nevertheless, with the justification of "supporting updated scientific medical education", the pharmaceutical companies focus mainly on the treating physicians (clinicians) by selecting medical "leaders", doctors who have influence among the medical groups or societies, or are representatives of health institutions. Whereas, the few scholars or scientists who are knowledgeable in the field of viral hepatitis are not considered under the argument that they are not clinicians or do not treat patients.

This situation leads to the lost opportunity to be supported by the pharmaceutical industry. Thus, in the absence of proposals and sanitary laws, the pharmaceutical industry is interested in selling their drug; but the academic or research sector in the developing countries do not fall within their scope.

RECOMMENDATIONS

HCV infection imposes a large challenge in the world, and it certainly will be eradicated faster in some regions than others. In low- and medium-income countries of LA the health problem of HCV may not depend entirely on money, other nations with fewer resources are proactively establishing public-private partnerships to lower the cost of the DDAs (*e.g.*, Egypt). Hopefully, these strategies will close the gap between the number of patients who are infected (diagnosed) versus those who are treated.

The advancement of scientific knowledge and its impact on health are correlated with the progressive changes in the attitude and behavior of key players and others responsible. The increase in knowledge should benefit all people irrespective of their socio-economic status. To achieve such impact, and to reach all those who need an efficient antiviral therapy, this change in attitude needs to become a reality.

Where and how to start to face this critical situation in Latin America? One recommendation is that both politicians and authorities must consider viral hepatitis as a health problem in their respective region, and establish support strategies to investigate the burden of HCV infection. It is no longer safe to assume that any health issue can be resolved without recognizing the magnitude of the situation.

In the medical communities, a good start would be to establish a partnership between the academic sector, researchers, and physicians, instead of independently acting on their own. This collaborative shift could strengthen the figure of the MD/PhD in each medical society. This may be achieved if alongside, the academic institutions in LA make every effort towards higher standards of education and professionalism. This would greatly strengthen the quality of the medical associations with academic leaders that contribute with knowledge publishable in indexed

international journals. In countries such as Spain and Brasil, the use of the h-index (Hirsch-index) has been considered to grant membership to their respective National Academy of Medicine.

Another important change would be to educate the younger generations that the purpose of belonging to a medical society is not only to be their president or chairperson. As mentioned above, the ultimate goals are to provide prestige and effectiveness by contributing to new knowledge about their country or city in matters of health. The higher income groups require treatments, however, so does the general population. If we reconsider and always remember the Hippocratic Oath, this will help to reflect and advance the field quickly to achieve these goals.

To conclude, the disclosure of the clinicians (speakers), in regards to their sponsorship by the pharmaceutical industry, should be regulated by law and enforced by an ethical practice. Likewise, academics, scientists, medical practitioners and the pharmaceutical companies should equally engage and commit to solving the problem of HCV infection in this region of the world. Moreover, grants given by the pharmaceutical industry in LA to support research in liver disease would be extremely beneficial. These are recommendations that need to be heavily considered by all key players.

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REVIEW

Epidemiology of hepatitis E virus in Iran

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Abstract

Iran is known as an endemic country for hepatitis E virus (HEV) infection, while there are variations in the epidemiology of HEV infection throughout the country. The available epidemiological studies in different regions of Iran show HEV seroprevalence of 1.1%-14.2% among general population, 4.5% -14.3% among blood donors, 6.1%-22.8% among injecting drug users, 6.3%-28.3% among hemodialysis patients, 1.6%-11.3% among patients infected with other hepatitis viruses, 27.5% among patients with chronic liver disease, 30.8% among kidney transplant recipient patients, and 10%-16.4% among human immunodeficiency virus-infected patients. These variations reflect differences in the status of public health and hygiene, risk factors, and routes of transmission in different regions and groups. Therefore, it is necessary to review the epidemiology of HEV infection to determine the most prevalent risk factors and routes of transmission, and to evaluate the effectiveness of preventive strategies employed in the public health services of the country. Moreover, the other epidemiological aspects of HEV, including the genotypic pattern, extra hepatic manifestations, and incidence of chronic infection need to be investigated among Iranian population to expand the current knowledge on the epidemiology of HEV and to clarify the real burden of HEV infection. Therefore, this review was performed to provide a general overview regarding the epidemiology of HEV in Iran.

Key words: Hepatitis E virus; General population; Blood donors; Injecting drug users; Hemodialysis; Immunocompromised patients; Chronic liver disease; Prevalence; Epidemiology; Iran

Core tip: Iran is considered as an endemic country for hepatitis E virus (HEV) infection, while there are variations in the epidemiology and prevalence of hepatitis E throughout the country. These variations reflect differences in the life styles, status of public health, risk factors, and routes of transmission in different groups and geographical regions of Iran.

Therefore, this study was conducted to review the epidemiological aspects of HEV infection in Iran.

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INTRODUCTION

Hepatitis E virus (HEV) is the causative agent of hepatitis E infection[1]. This infection is usually asymptomatic or acute self-limiting[1] but might lead to fulminant hepatitis or even long-term chronic infection and cirrhosis with a high rate of mortality due to severe liver failure in high-risk groups, such as pregnant women, organ transplant recipient patients, immunocompromised patients, and those with pre-existing liver problems[1-6]. HEV is predominantly transmitted *via* the fecal-oral route; however, transmission of HEV through blood transfusion, organ transplantation, hemodialysis, placenta and sexual intercourse is also possible[1,7,8]. Among these, sexual transmission of HEV is less common and has mostly been reported in homosexual males[7,9].

HEV is a small spherical virus with a positive-sense RNA genome and an icosahedral non-enveloped capsid[5,10-12]. The viral genome contains three partially overlapping open reading frames[5,13,14]. HEV has been classified into the family *Hepeviridae*, the genus *Orthohepevirus*, and the species *Orthohepevirus A*[1,15]. There are four genotypes of HEV capable of causing human infection with different epidemiological features[3,16]. These genotypes have further been subdivided into 24 subgenotypes[5,17,18]. Genotypes 1 and 2 are only recognized in human beings, while genotypes 3 and 4 are found in domestic and wild animals as well[7,19-21]. Despite this genetic heterogeneity, only one serotype has been recognized so far[1,5,22].

HEV genotypes differ in their severity, pathogenecity, mortality rates, mode of transmission, age distribution, and geographical distribution[1,7-9,23]. HEV genotype 1 is frequently found in North Africa and Asia[9]. HEV genotype 2 is more common in West Africa and Mexico[7,9]. HEV genotype 3 is considered to have a worldwide distribution and is more prevalent in several European and American countries, as well as Japan, China, Australia, and New Zealand[7,9,10]. HEV genotype 4 has been reported in Asian countries and more recently in Central Europe[5,9,23]. Genotypes 3 and 4 appear to be less virulent than genotypes 1 and 2[1,24]. HEV genotype 1 is associated with the most cases of fulminant hepatitis and high mortality during pregnancy[8,10]. While HEV genotype 3 is the cause of almost all cases of chronic HEV infections worldwide[5,7,8]. Infection with HEV genotype 4 seems to be asymptomatic and mostly remains undiagnosed[6,9].

Hepatitis E is usually an asymptomatic or acute self-limited infection and only requires supportive care. However, when fulminant or chronic hepatitis arises, therapeutic intervention is an obligation[9,10]. Reduction of immunosuppressive therapy is considered as the first-line therapy in immunocompromised patients with chronic HEV infection. Nevertheless, at the same time, it can increase the risk of graft rejection[3,5,9,25-27]. In such conditions, organ transplant recipient patients benefit most from antiviral therapy, including pegylated interferon (peg-IFN) monotherapy or combination therapy with ribavirin and peg-IFN[3,10,25].

These antiviral agents are associated with severe side effects. IFN-therapy may result in acute graft rejection[3,5,8,25]. Ribavirin administration induces severe hemolytic anemia; and when the dose of ribavirin is reduced, the viral clearance is not achieved[8]. Therefore, the combination of ribavirin with a reduction in the doses of immunosuppressive drugs has been found to be the most promising treatment option[5,10,25]. In addition, these antiviral drugs should be administered with caution during pregnancy due to their teratogenicity[10,27,28]. Early delivery of fetus or termination of pregnancy should be considered as another option to save mothers' lives[28,29]. Administration of the antiretroviral drugs in human immunodeficiency virus (HIV)-positive patients can result in an increase in the proportion of T helper cells and subsequently clearance of HEV infection[25].

Considering the side effects and limitations of the currently available treatment regimens as well as the absence of a specific antiviral treatment for HEV infection, preventive measures and vaccination against HEV infection are the most desirable approaches for controlling HEV infection[25,30]. HEV vaccines using the truncated forms of capsid protein have been evaluated in human clinical trials, and one of them, HEV 239 vaccine, has been approved in china in 2011. Although this vaccine has shown promising results, it is not commercially available worldwide[5,20,27,31]. Therefore, preventive measures are still known to be the best option. Providing clean drinking water supplies, improving the hygienic infrastructure and sanitary status, washing hands and vegetables properly, boiling drinking water, and avoiding consumption of undercooked foods and unpeeled fruits are some of these preventive measures[1,5,11,23,31,32]. In addition, proper chlorination of water supplies, sanitary preparation of food, and public awareness regarding the possible routes of HEV transmission are essential to reduce the risk of exposure to HEV in the community[1,11,23].

HEV has infected one-third of the world's population[13,17]. In addition, 20 million new cases and 3.3

million acute cases of HEV infection occur globally each year[3,33], and HEV-related hepatic failure is responsible for approximately 56600 deaths per year[33,34]. The mortality rate of HEV infection is 1%-2% in the general population[17], but it may rise to 10%-25% in pregnant women[7,20] and over 75% in individuals with pre-existing liver problems[20].

HEV is a considerable global health concern. Although HEV infection was traditionally believed to be limited to developing countries, currently it is known that this infection has a worldwide distribution with different epidemiological patterns[9]. In developing countries where the infection is endemic, acute outbreaks or large epidemics of hepatitis E occur due to contamination of water supplies mainly at the time of heavy rainfall or following floods. These outbreaks are largely due to genotypes 1 and 2 and more frequently affect young adult males[1,3,5,8,9,18]. Whereas in developed countries, HEV infection is non-endemic and mostly occurs as sporadic locally-acquired disease due to consumption of contaminated food supplies. The infection is due to genotypes 3 and 4 and predominantly found among middle-aged elderly males throughout the year[1,3,5,9,18].

Apart from variations in the global epidemiological patterns of HEV, there is a wide range of variation in the prevalence and epidemiology of HEV infection within a country[5,16,35,36]. These variations reflect differences in the lifestyle, status of public health, risk factors, and routes of transmission in different regions and groups[35-37]. It is therefore necessary to review the epidemiology of HEV infection to determine the most prevalent risk factors and routes of transmission and to evaluate the effectiveness of prevention strategies employed in the public health services of a country. These epidemiological studies are not only essential for improving the current strategies to minimize the risk of acquiring HEV infection in the society but also clarify the real burden of HEV infection.

This study has the objective to review the epidemiology of hepatitis E in Iran, a vast country located in the Middle East, with an extension of about 1700000 km^2 and an estimated population of 70 million inhabitants in different provinces with various ethnicities[38].

HEV IN GENERAL POPULATION

With an overall prevalence rate of more than 5% in the general population, Iran is considered as an endemic country for HEV infection[36,39]. However, the seroprevalence of hepatitis E in the general population varies considerably in different parts of the country, ranging from 0.0% to 0.9% for anti-HEV IgM and 1.1% to 14.2% for anti-HEV IgG and the total HEV antibodies in different studies[35,39-46] (Table 1[35,39-46]). Differences in the lifestyles, risk factors, levels of exposure, the geographic regions, study population, study periods, sample sizes, time of sampling, and the diagnostic accuracy of kits used to determine anti-HEV antibodies in various studies can explain these variations[35-37]. However, the role of public health services, hygienic conditions, socioeconomic status, and environmental factors should not be dismissed[36,37,42].

The predominant mode of transmission in Iran is fecal-oral route, especially feces-contaminated drinking water; however, the other routes of transmission might also have a minor role in the spread of HEV but with undetermined importance[35,43,47]. Food-borne transmission of zoonotic origin is unlikely, since wild animal hunting and swine farming are prohibited in Iran[35]. Person-to-person transmission is most likely rare in Iran, since no association between the household size and seroprevalence rate of HEV has been reported[42]. Overall, the importance of the other probable routes of transmission in spread of HEV infection in the society still requires to be determined.

Despite the epidemiological pattern in developing countries, where HEV most often affects young adults[1,5,9], the seroprevalence rate in Iran increases with age due to cumulative exposure to HEV over time with the highest prevalence rate among middle-aged and elderly individuals aged over 50 years[35,36,40,41,45]. Another possible reason is improvement in public hygiene, sanitation, sewage disposal and drinking water supply systems, which has resulted in decreased prevalence of HEV infection in young population of the country over time[36,47].

Except two reports[44,45], in the majority of studies, the seroprevalence of HEV was higher in females compared to males. However, none of these differences were significant[35,36,39-42]. In rural areas, inhabitants were more likely to be positive for HEV serological markers compared to individuals living in urban areas[39,43]. Appropriate access to the public health services, sewage disposal systems, and safe water supplies in cities can explain these differences in the seroprevalence rates regarding the place of residence[35,37,43]. Taken together, in studies from Iran, socioeconomic status, level of sanitation, population density, age, level of education, and place of residency were found to be risk factors for acquiring HEV infection in the society[35,42,43,48].

In one study from Iran, the prevalence of anti-HEV IgG antibody in the general population was as high as 46.1% in South-West of the country[36]. The reason of this high endemicity is most likely Karun River as the drinking water source of the inhabitants, where the city sewage is also discharged in. It is also worthy to note that the participants in the mentioned study were all adults, mostly middle-aged and elderly adults[36]. Iran is located in the Middle East between HEV high endemic countries on the eastern and western borders. This considerable geographic location has affected epidemiological pattern of HEV infection in Iran[35,47].

A few occasional waterborne outbreaks of hepatitis E have also occurred in Iran[47]. The first documented outbreak was reported in Kermanshah city, West of

Table 1 Seroprevalence of hepatitis E virus in different population groups in Iran

Study population	City or province	Location	Year of study	No. of participants	Age, mean ± SD (age group), yr	No. of positive cases	HEV seroprevalence	HEV diagnosis	Manufacturer of Serology kits	Ref.
General population	Nahavand	West	2003	1824	34.7 ± 19.5 (6 to > 70)	170	9.3%	Anti-HEV IgG	DIA.PRO, Italy	Taremi *et al*[42]
General population	Sari, Mazandaran	North	2003	1080	2 to 25	25	2.3%	Anti-HEV IgG	DIA.PRO, Italy	Saffar *et al*[43]
General population	Tehran and Golestan	North-Center North-East	2006	1423	37.9 ± 13.4 (18 to 65)	105	7.4%	Anti-HEV total antibodies	DIA.PRO, Italy	Sepanlou *et al*[39]
General population	Shiraz	South	2011-2012	1030	< 1 to 95	138	13.4%	Anti-HEV total antibodies	DIA.PRO, Italy	Asaei *et al*[40]
						9	0.9%	Anti-HEV IgM		
General population	Mashhad	North-East	2009	1582	29.06 ± 18.513 (1 to 90)	225	14.2%	Anti-HEV total antibodies	DIA.PRO, Italy	Ahmadi Ghezeldasht *et al*[35]
General population	Isfahan	Center	2005	816	6 to > 50		3.8%	Anti-HEV total antibodies	DIA.PRO, Italy	Ataei *et al*[41]
General population	Tehran	North-Center	2006-2007	551	41.28 ± 16.96 (1 to 83)	51	9.3%	Anti-HEV IgG	DIA.PRO, Italy	Mohebbi *et al*[44]
General population	Ahvaz	South-West	2014	510	45.89 ± 14.63 (18 to 81)	235	46.1%	Anti-HEV IgG	DIA.PRO, Italy	Farshadpour *et al*[36]
						7	1.4%	Anti-HEV IgM		
General population	Khorramabad	West	2009	400	36 (> 20)	31	7.8%	Anti-HEV total antibodies	(ND)	Raoofi *et al*[45]
Soldier	Tehran	North-Center	2006	800	19 ± 1.2 (17 to 23)	9	1.1%	Anti-HEV IgG	DIA.PRO, Italy	Ghorbani *et al*[46]
							0.0%	Anti-HEV IgM		
Blood donor	Khuzestan	South-West	2005	400	33.3 (18 to 60)	46	11.5%	Anti-HEV IgG	HEV-EIA, Biokit, Spain	Assarehzadegan *et al*[55]
Blood donor	Tehran	North-Center	2003-2004	90	31.8 ± 11	7	7.8%	Anti-HEV total antibodies	DIA.PRO, Italy	Aminiafshar *et al*[54]
Blood donor	Tabriz	North-West	2004	399	31.4 ± 9.8	31	7.8%	Anti-HEV IgG	DIA.PRO, Italy	Taremi *et al*[49]
Blood donor	Markazi	West-Center	2012	530	36.3 ± 11.7 (18 to 71)	76	14.3%	Anti-HEV IgG	DIA.PRO, Italy	Ehteram *et al*[53]
Blood donor	Kerman	South-East	2007-2008	400	20 to 60	31	7.7%	Anti-HEV IgG	DIA.PRO, Italy	Arabzadeh *et al*[52]
Blood donor	Tehran	North-Center	2014	559	38 (18 to > 47)	45	8.1%	Anti-HEV IgG	DIA.PRO, Italy	Hesamizadeh *et al*[50]
Blood donor	Tehran	North-Center	ND	200	20 to 61	9	4.5%	Anti-HEV antibodies	DRG, Diagnostics, Germany	Keyvani *et al*[51]
Drug users (addicts)	Hamadan	West	2011-2012	131 (IDUs)	35.57 ± 8.13 (22 to 70)	8	6.1%	Anti-HEV IgG	DIA.PRO, Italy	Keramat *et al*[58]
				131 (non-IDUs)	31.57 ± 8.19 (20 to 45)	2	1.5%			
Drug users	Ahvaz	South-West	2005-2006	228	34.1 ± 6.1 (18 to 54)	35	15.4%	Anti-HEV IgG	DIA.PRO, Italy	Alavi *et al*[57]
				114 (IDUs)		26	22.8%			
				66 (Inhalant)		6	9.1%			
				48 (Oral opiate)		3	6.2%			
Hemodialysis	Hamadan	West	2010	153	< 20 to > 60	30	19.2%	Anti-HEV IgG	DIA.PRO, Italy	Eini *et al*[63]
Hemodialysis	Tabriz	North-West	2004	324	53.5 ± 15.1	24	7.4%	Anti-HEV IgG	DIA.PRO, Italy	Taremi *et al*[64]
Hemodialysis	Jahrom	South	2007	43	59.3 ± 14.4	3	7.0%	Anti-HEV IgG	DIA.PRO, Italy	Pourahmad *et al*[65]
Hemodialysis	Zanjan	West	2011	93	57.0 ± 18.5 (16 to 88)	25	26.9%	Anti-HEV total antibodies	DIA.PRO, Italy	Mobaien *et al*[66]
Hemodialysis	Jahrom and Shiraz	South	2010	80	55.69 ± 14.70 (26 to 80)	5	6.3%	Anti-HEV IgG	DIA.PRO, Italy	Zekavat *et al*[67]
Hemodialysis	Ahvaz	South-West	ND	47	55.27 ± 8.1	5	10.6%	Anti-HEV IgG	DIA.PRO, Italy	Beladi Mousavi *et al*[68]
Hemodialysis	Isfahan	Center	2012	274	59.9 ± 16.4 (21 to 80)	78	28.3%	Anti-HEV IgG	DIA.PRO, Italy	Alavian *et al*[69]
HCV-infected patients	Tehran	North-Center	ND	100	20 to 61	7	7%	Anti-HEV antibodies	DRG, Diagnostics, Germany	Keyvani *et al*[51]
HBV-infected patients	Tehran	North-Center	ND	150	20 to 61	17	11.3%	Anti-HEV antibodies	DRG, Diagnostics, Germany	Keyvani *et al*[51]
Thalassemia patients with chronic hepatitis C	Iran	Iran	2009-2010	64	25.08 ± 6.46 (12 to 76)	1	1.6%	Anti-HEV IgG	DIA.PRO, Italy	Karimi Elizee *et al*[56]

Hemophilia patients with chronic hepatitis C	Iran	Iran	2009-2010	155	30.63 ± 11.51 (12 to 76)	5	3.2%	Anti-HEV IgG	DIA.PRO, Italy	Karimi Elizee *et al*[56]
GB Virus C positive hemodialysis patients	Gorgan	North-East	2012	22	54.32 ± 12.56	0	0.0%	total anti-HEV	DIA.PRO, Italy	Kelishadi *et al*[77]
patients with chronic liver disease	Azerbaijan	North-West	2005-2006	200	48.26 ± 18.19 (10 to 87)	55	27.5%	Anti-HEV IgG	DIA.PRO, Italy	Somi *et al*[78]
HIV-infected patients	Tehran	North-Center	2012	100	38	10	10%	Anti-HEV IgG	DIA.PRO, Italy	Ramezani *et al*[79]
						0	0.0%	Anti-HEV IgM		
						0	0.0%	HEV RNA		
HIV-infected patients	Shiraz	South	2013	158	39.1 ± 8	26	16.4%	Anti-HEV total antibodies	DIA.PRO, Italy	Joulaei *et al*[80]
Kidney transplant recipient patients	Urmia	North-West	1991-2010	91	35.4 ± 14.5 (6 to 65)	28	30.8%	Anti-HEV IgG	DIA.PRO, Italy	Rostamzadeh Khameneh *et al*[81]

ND: Not defined; HEV: Hepatitis E virus.

Iran, in 1991. At the same time, a suspected outbreak was reported in Isfahan province, during which over 100 inhabitants were infected in Fereidon-Shahr. Another outbreak occurred in Lordegan, Southwest of Iran, in 1999 and affected 154 people[45,47]. The history of these outbreaks clearly implies that HEV infection is not new to Iran, and probability of future outbreaks in the country should be considered and preventive strategies for controlling transmission of HEV infection should be provided.

The prevalence rate of HEV infection in the general population is likely underestimated due to the lack of adequate population-based studies, asymptomatic nature of HEV infection, and the fact that hepatitis E is not a reportable infection in the public health system of Iran. In addition, the incidence and case fatality rate of hepatitis E in the general population of Iran are unclear and need further investigation.

HEV IN BLOOD DONORS

Even though HEV infection is an old enterically transmitted disease, it is also considered an emerging transfusion-transmitted infection[4,7]. Since only recently, HEV has been recognized as a threat to blood safety[4,6]. The possibility of HEV transmission through blood transfusion dates back to 2002 and 2004, when two molecular studies in Japan introduced HEV as a transfusion transmissible virus[6,7,18]. Since then, several studies from Japan, the United Kingdom, France, and Saudi Arabia have confirmed HEV transmission through blood transfusion[6,7,18,23].

These studies have reported high prevalence of anti-HEV antibodies, and viral RNA among blood donors[6,23]. Donor-recipient linked studies have also confirmed transmission of HEV to blood transfusion recipients[6,7]. In addition, higher incidence of hepatitis E in multi-transfused individuals compared to controls suggests this transmission[7,18,23]. Moreover, the high rate of asymptomatic or undiagnosed infection among blood donors increases the risk of HEV transmission[7,18,23]. Fortunately, these unnoticeable infections are preventable through screening of blood donations for HEV infection. Screening methods are based on the detection of anti-HEV antibodies and HEV RNA in serum or plasma samples of blood donors[1,4,7,8,16,18].

The presence of elevated liver enzymes and HEV RNA in blood is short lived, and becomes normalized or undetectable approximately 6 weeks and 3 weeks after the onset of clinical illness, respectively[5,7,16]. In some instances, viremia may persist for a longer period, especially in children after acute hepatitis E[4,7]. In addition, HEV RNA has been detected up to 3 years in immunocompromized patients, especially those with renal transplantation[4]. IgM increases during the acute phase of infection and becomes undetectable after 3-8 mo[16]. While IgG appears after the increase of IgM level and persists for years with unknown duration[5,8,16]. Therefore, anti-HEV IgG positive samples in the absence of IgM and HEV RNA are defined as past HEV infection. While a positive anti-HEV IgM test can be indicative of current

infection if HEV RNA is detected[1,16]. Some patients in viremic phase of infection do not show anti-HEV IgM responses[49]. Majority of risks are due to the presence of HEV RNA in blood of apparently healthy donors with normal levels of liver enzymes and negative anti-HEV IgM, which is indicative of asymptomatic viremia[7,18]. In these instances, blood transfusion is capable of trasmitting HEV infection despite negative serological markers. Therefore, HEV is a potential threat to blood safety[7,18].

Since 2005, Japan has implemented HEV RNA testing of all donors along with screening for elevated liver enzymes levels[7]. While some other countries perform selective HEV screening for high-risk recipients[8]. The necessity to screen all blood donations or at least a part of them for HEV infection needs to be considered in Iran.

Studies on the seroprevalence of HEV in blood donors are limited in Iran and mainly have been conducted in main cities, while the seroprevalence varies from 4.5% to 14.3% in these studies[49-55] (Table 1[49-55]). Despite this high prevalence, screening of blood donors for HEV is not performed in the blood banks of Iran until more evidence becomes available regarding the potential threat of HEV to blood safety[49,53]. In addition, the risk of incidence and transmission of hepatitis E by blood transfusion in Iran is unknown. Since the studies in Iran have only reported the rate of seropositivity, which is incapable of estimating the rate of viremic blood donors[49,53]. Therefore, additional studies to investigate the possibility of HEV transmission through blood transfusion seem to be necessary. Even if the risk of transmission through blood transfusion is low, we should not neglect the importance of this infection. Since HEV causes serious consequences in high-risk recipients, who often require blood transfusion. Therefore, access to HEV-free blood and blood products is the highest priority for this group of patients[4,7,18,50].

The seroprevalence of HEV among hemophilia and thalassemia patients in Iran is lower than expected and is in the range of that found in the general population of Iran[56]. The reason of this low prevalence may be that the blood donor population in Iran has mostly been consisted of young individuals, while HEV mostly affects middle-aged or old population in Iran[49,50,54]. This suggests that blood transfusion may not be a risk factor for transmission of HEV infection among hemophilia and thalassemia patients in Iran[56]. Still, more studies are required to confirm this issue.

HEV IN INJECTING DRUG USERS

With having approximately 180000 injecting drug users (IDUs) among Iranian adults aged 15-64 years, Iran is considered one of the countries with the highest numbers of injection drug users in the world[38]. While only two studies have assessed the possible effect of injecting drug use on the seroprevalence of HEV among IDUs in Iran[57,58]. Alavi *et al*[57] reported high seroprevalence of HEV in IDUs (22.8%) compared to inhalant drug users (9.1%) and oral opiate drug users (6.2%) and suggested an association between injection drug abuse and HEV seropositivity in Ahvaz in 2005-2006. While some other studies from France, the United States (US), and Denmark have rejected this association[59-62]. Keramat *et al*[58] reported high prevalence of HEV in IDUs (6.1%) compared to non-IDUs control group (1.5%) and found no relationship between duration of injection and HEV seroprevalence in Hamadan in 2011-2012.

These studies indicated high seroprevalence of HEV infection among IDUs in Iran[57,58], while this seroprevalence was not influenced by the type of substance abused but was associated with the route of administration[57]. According to the results of these studies, IDUs in Iran are at risk of acquiring HEV infection most likely due to exposure to infected blood through sharing syringe[58]. As a result, injection drug use was proposed as a possible route of HEV transmission. However, still more investigations are required to confirm this issue.

HEV IN HEMODIALYSIS PATIENTS

The seroprevalence of hepatitis E among patients on maintenance hemodialysis (HD) varies considerably from 4% to 28.3% in different cities of Iran[63-69] (Table 1[63-69]). The reason of this vast geographic variation in different HD centers is unknown, but it may be due to the different levels of safety strategies in HD units, as well as the public health and prevalence of HEV infection in the community[30,64,69]. In some studies, HEV seroprevalence in HD patients is lower than or in the range of HEV seroprevalence in the general population of Iran[64,65,67], indicating a low risk of exposure to HEV in these areas or maybe a negligible HEV transmission in HD centers. While in some other studies, it is noticeably higher than HEV seroprevalence in the general population, which may be indicative of parenteral transmission of HEV infection[30,63,66,69]. Similar high prevalence of anti-HEV antibodies in HD patients has been reported in Egypt (22.9%)[70], Japan (30%)[71], and Turkey (20.6%)[72]. In contrast, reports from Italy (6.0%)[73], Brazil (6.2%)[74], and Spain (6.3%)[75] have indicated a low seroprevalence of HEV among HD patients.

The seroprevalence of HEV infection among Iranian HD patients was associated with almost no risk factor in most of these studies[63,64,67,68]. While duration of HD was significantly associated with HEV seropositivity in two studies[65,69]. In addition, in one study, 41.7% of HEV seropositive HD patients had a history of blood transfusion[69]. These studies support the nosocomial transmission of HEV infection[30,47,65]. While the others indicate a rare acquisition of HEV infection through hemodialysis[64,67]. Overall, the epidemiology of HEV infection among HD patients in Iran seems to be

a controversial issue due to these variations in the results of so far conducted studies. Therefore, more extensive or comprehensive studies in different geographical regions of Iran are required to resolve these conflicts between the results and to determine the exact epidemiological pattern of HEV infection among HD patients.

Considering the clearance of a significant level of anti-HEV antibodies during the process of dialysis as well as weak antibody responses due to chronic renal disease, a considerable proportion of HEV seropositive HD patients may be reported as seronegative[30,67,69,76-81]. Except one study[65], the serum levels of liver enzymes were normal or low in HEV seropositive patients on maintenance hemodialysis due to the fast reduction of these enzymes to the normal levels[30,67]. Therefore, some HEV-infected HD patients may remain undiagnosed. These HD patients with inapparent HEV infection might be the main source of HEV transmission as a nosocomial infection in HD units[30]. Overall, neither anti-HEV antibodies nor level of liver enzymes can be valid diagnostic markers in case of HEV infection among HD patients[30]. Therefore, serious safety measures and proper screening of HD patients for hepatitis E in HD centers should be considered to prevent transmission of HEV during the process of hemodialysis.

HEV IN PATIENTS INFECTED WITH OTHER HEPATITIS VIRUSES

Viral hepatitis infections are believed to be associated with an increased risk of hepatitis E occurrence, and co-infection or superinfection with HEV will enhance the risk of liver failure[11,17,82,83]. In recent years, several studies have reported high prevalence of hepatitis E among patients with chronic viral hepatitis and supported the possibility of parenteral transmission of HEV[30,82,84], while other studies have demonstrated a low occurrence of these co-infections or superinfections and found no association between HEV and other viral hepatitis[85,86]. This variation in the prevalence of HEV among patients with other viral hepatitis reflects differences in the routes of transmission and distribution of these hepatotropic viruses in different parts of the world. Although HEV is predominantly transmitted *via* the fecal-oral route, the possibility of parenteral transmission has also been reported in endemic countries[82,87].

Currently, only a few reports are available regarding the prevalence of HEV infection among patients with viral hepatitis in Iran. Keyvani *et al*[51] reported high prevalence of anti-HEV antibody in HBV (11.3%) and HCV (7%)-infected patients compared to healthy blood donors (4.5%) in Tehran. In another study by Karimi Elizee *et al*[56], the seroprevalence of HEV among thalassemia and hemophilia patients with chronic hepatitis C was reported to be 1.6% and 3.2%, respectively, which is similar to HEV seroprevalence in Iranian general population. Kelishadi *et al*[77] reported the absence of anti-HEV IgG antibody in GB virus C positive hemodialysis patients in Gorgan. These studies were unable to determine the effect of hepatitis E on the clinical outcomes of the other viral hepatitis. Overall, data concerning dual infection with hepatitis E and the other viral hepatitis in Iran are scarce, and the routes of HEV transmission in this group of patients are unclear. Therefore, further studies are required to determine the association between HEV and other viral hepatitis in Iran.

HEV IN IMMUNOCOMPROMISED AND IMMUNOSUPPRESSED PATIENTS

HEV infection in immunocompromised and immunosuppressed patients may lead to chronic hepatitis E, with an increased risk of developing liver fibrosis and cirrhosis, and subsequently lower survival of the infected patients[9,10,27]. Chronic HEV infection is characterized by the persistent presence of detectable HEV-RNA in serum and stool for more than 6 mo (more than 3 mo in organ transplant recipient patients) along with persistently elevated liver enzymes[3,5,8,9,33]. So far, chronic hepatitis E has been observed in HIV-infected patients, organ transplant recipient patients, and those with hematological malignancies, who receive anticancer chemotherapy[5,10,25-27,88]. However, the possibility of HEV chronicization in other patients with immunosuppressive conditions is currently under investigation, and this chronic infection may identify in more categories of patients in near future[3].

More recently, some cases of chronic HEV infection have also been observed in elderly immunocompetent individuals[8]. While no report of chronic infection has been documented in pregnant women and infants[16,28]. Almost all cases of chronic hepatitis E have been observed following infection with HEV genotype 3[3,5,7,25]. The first case of chronic hepatitis E caused by HEV genotype 4 has recently been identified in a Chinese patient[3,9].

The seroprevalence of hepatitis E among organ transplant recipient patients varies from 2.3% to 43.9% in different studies[5]. While the prevalence of HEV infection based on the detection of viral RNA ranges from 0.9% to 3.5%[5]. This prevalence among transplant recipient patients with elevated liver enzymes is 4.3%-6.5%[5]. The chronicity rate of hepatitis E is approximately 60% in organ transplant recipient patients without therapeutic interventions[25,26,30].

Indeed, progression to chronicity in immunocompromised patients could be mediated by inability to clear the virus after acute infection, which is related to the degree of immunosupression and immunological status of transplant recipient patients at the time of HEV infection as well as the time period between the transplantation and incidence of HEV infection[3,5,10,29].

Therefore, suboptimal HEV-specific cellular immune responses, low lymphocyte and platelet counts, the occurrence of HEV infection immediately after transplantation, and the use of more effective immunosuppressive drugs such as tacrolimus are risk factors for the incidence of chronic hepatitis E in immunocompromised patients following exposure to HEV[1,3,5,26,88]. Even the presence of anti-HEV IgG antibodies prior to re-exposure to HEV cannot exclude the chance of reinfection in transplant recipient patients, and such reinfections may lead to chronic infection[3]. The main route of HEV transmission in immunocompromised patients seems to be fecal-oral, especially *via* consumption of contaminated food[3,5,16]. However, acquisition of HEV infection following blood transfusion and liver transplantation is also possible but seems to be uncommon[3,5,26]. Most patients with chronic hepatitis E are asymptomatic, and the rest show nonspecific symptoms, including fatigue, fever, abdominal pain, asthenia, and very rarely jaundice[5,25]. Chronic hepatitis E can rapidly progress to liver fibrosis, cirrhosis, and subsequently fatal liver failure in immunocompromised patients[3,5,25]. In addition, numerous hepatitis E-associated extrahepatic manifestations, including neurological, hematological, musculoskeletal, renal manifestations, as well as acute pancreatitis, autoimmune thyroiditis, myocarditis, mixed cryoglobulinemia, thrombocytopenia, arthralgia, Henoch-Schonlein purpura, myasthenia gravis, haemolysis, membranous glomerulonephritis associated with immunological disorders and many others have been reported in patients with acute or chronic HEV infection[7,9,25,33,89,90].

Such extrahepatic complications sometimes outshine clinical manifestations of hepatic injury, and the causative agents, hepatitis E, might not be suspected. Therefore, the probability of hepatitis E in extrahepatic manifestations should be considered[33].

Chronic hepatitis E results in graft loss and subsequently retransplantation in organ transplant recipient patients. However, recurrent hepatitis E and subsequently progressive chronic infection after retransplantation may also occur if the viral clearance is not achieved before retransplantation[5].

In this situation, early diagnosis of hepatitis E in this group of patients is the highest priority. The diagnosis should be based on the detection of HEV RNA in serum, cerebrospinal fluid (CSF) in case of neurological complications or stool samples, not levels of liver enzymes and results of serological tests[3,26,33,91]. Since various factors can elevate liver enzymes, including drugs, toxin, graft rejection, infections, and biliary tract dysfunction[30]. Furthermore, the presence of chronic HEV infection in organ transplant recipient patients is sometimes accompanied by normal liver enzymes[30]. In addition, the delay or absence of seroconversion and loss of anti-HEV antibodies are frequently observed in this group of patients due to immunosuppressive conditions, which result in suppression of antibody development over time[5,91]. Therefore, immunocompromised or immunosuppressed patients with chronic HEV infection may have normal liver enzymes and negative serological tests[26,30,81].

In these conditions of uncertainty, the awareness of physicians regarding chronic HEV infection is crucial. Since most cases of chronic HEV infection may be missed due to the lack of HEV consideration among physicians or inappropriate choose of diagnostic assays[3,25].

Reports on HEV prevalence in immunocompromised patients in Iran are scarce. Rostamzadeh Khameneh *et al*[81] assessed the seroprevalence of HEV among 91 Iranian kidney transplant recipient patients. Overall, the seroprevalence of HEV was 30.8%. Joulaei *et al*[80] reported a HEV seroprevalence of 16.4% among 158 HIV-infected individuals in Shiraz in 2013. In another study by Ramezani *et al*[79], the seroprevalence of HEV infection was found to be 10% among 100 HIV-positive individuals in Tehran in 2012. These limited studies were unable to determine the incidence and prevalence of chronic HEV infection among immunocompromised patients in Iran. Since only the seroprevalence of anti-HEV IgG antibodies has been assessed, while HEV-RNA has not been measured in these studies[81,88]. Therefore, more studies are required to gain insight into the burden of chronic HEV infection in Iran.

CONCLUSION

However, Iran is classified as an endemic region for HEV infection, but we do not know much about this infection in Iran. The available epidemiological data have demonstrated the seroprevalence of HEV infection in different groups and regions of Iran, while the presence of HEV-RNA has not been evaluated in the studies published so far. In addition, the distribution pattern of HEV genotypes is unknown in Iran.

From historical aspect, hepatitis E is not new in Iran but is underestimated due to the lack of awareness amongst physicians and inappropriate diagnosis of the infection. The importance of HEV infection as a main public health problem cannot be neglected any longer. The identification of HEV-associated extrahepatic manifestations and chronic hepatitis E in immunocompromised patients has attracted attention to the study of HEV in recent years. While these new aspects of so thought acute self-limited hepatitis remain unknown in Iran. Overall, still a long way is ahead to determine the epidemiological patterns of HEV in Iran. To approach this goal, further epidemiological investigations at the national level are needed to more clearly delineate the incidence and prevalence of HEV infection in Iran. In addition, nationwide efforts should be pursued to control and prevent HEV infection in Iran.

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ORIGINAL ARTICLE

Basic Study

Structural and molecular features of intestinal strictures in rats with Crohn's-like disease

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Author contributions: Talapka P and Berkó A contributed equally to this work; Talapka P performed the majority of experiments and analyzed the data; Berkó A and Nagy LI performed the molecular investigations; Chandrakumar L and Bódi N participated equally in treatment of animals; Bagyánszki M, Puskás LG, Fekete E and Bódi N designed and coordinated the research; Talapka P, Fekete E and Bódi N wrote the paper; Talapka P and Berkó A contributed equally to this work.

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Abstract

AIM: To develop a new rat model we wanted to gain a better understanding of stricture formation in Crohn's disease (CD).

METHODS: Chronic colitis was induced locally by the administration of 2,4,6-trinitrobenzenesulfonic acid (TNBS). The relapsing inflammation characteristic to CD was mimicked by repeated TNBS treatments. Animals were randomly divided into control, once, twice and three times TNBS-treated groups. Control animals received an enema of saline. Tissue samples were taken from the strictured colonic segments and also adjacent proximally and distally to its 60, 90 or 120 d after the last TNBS or saline administrations. The frequency and macroscopic extent of the strictures were measured on digital photographs. The structural

features of strictured gut wall were studied by light- and electron microscopy. Inflammation related alterations in TGF-beta 2 and 3, matrix metalloproteinases 9 (MMP9) and TIMP1 mRNA and protein expression were determined by quantitative real-time PCR and western blot analysis. The quantitative distribution of caspase 9 was determined by post-embedding immunohistochemistry.

RESULTS: Intestinal strictures first appeared 60 d after TNBS treatments and the frequency of them increased up to day 120. From day 90 an intact lamina epithelialis, reversible thickening of lamina muscularis mucosae and irreversible thickening of the muscularis externa were demonstrated in the strictured colonic segments. Nevertheless the morphological signs of apoptosis were frequently seen and excess extracellular matrix deposition was recorded between smooth muscle cells (SMCs). Enhanced caspase 9 expression on day 90 in the SMCs and on day 120 also in myenteric neurons indicated the induction of apoptosis. The mRNA expression profile of TGF-betas after repeated TNBS doses was characteristic to CD, TGF-beta 2, but not TGF-beta 3 was up-regulated. Overexpression of MMP9 and down-regulation of TIMP1 were demonstrated. The progressive increase in the amount of MMP9 protein in the strictures was also obvious between days 90 and 120 but TIMP1 protein was practically undetectable at this time.

CONCLUSION: These findings indicate that aligned structural and molecular changes in the gut wall rather than neuronal cell death play the primary role in stricture formation.

Key words: Crohn's disease; Rat model; TGF-beta; Intestinal strictures; MMP9; TIMP1

Core tip: Intestinal strictures in Crohn's disease (CD) cause hardly treatable complications in patients. The aim of this study was to find the correlation between the intestinal stricture formation, the damaged innervation of smooth muscle cells (SMCs) and the changed expression of TGF-beta 2, 3 and MMP9/TIMP1 in rats with CD by using different light- and electron microscopic and molecular biological methods. Our findings indicate that disintegration of SMCs due to the up-regulation of TGF-beta 2 and off-balance in MMP9/TIMP1 expression rather than neuronal cell death play the primary role in the formation of intestinal strictures in CD.

Talapka P, Berkó A, Nagy LI, Chandrakumar L, Bagyánszki M, Puskás LG, Fekete E, Bódi N. Structural and molecular features of intestinal strictures in rats with Crohn's-like disease. *World J Gastroenterol* 2016; 22(22): 5154-5164 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i22/5154.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i22.5154

INTRODUCTION

Despite the fact that the formation of obstructive strictures is the leading cause of surgical intervention in patients with Crohn's disease (CD), little is known about their etiopathogenesis, and no direct therapies are available for the effective prevention or reversal of this condition[1]. Lasting deep remission has emerged as a major therapeutic goal in CD[2,3]. This implies not only alleviation of the symptoms, but also the achievement of complete mucosal healing, with the accompanying decrease in the risk of irreversible pathological alterations of the gut wall[4]. However, total mucosal regeneration to prevent stricture formation is unattainable[5,6].

The spread of fibrosis deep into the gut wall leads to disorganization of the lamina muscularis mucosae (LMM) and thickening of all layers of the gut wall due to the accumulation of extracellular matrix (ECM) elements[5,6]. Previous studies have demonstrated that the cytokines transforming growth factor-beta (TGF-β) isoforms and the tissue-degrading matrix metalloproteinases (MMPs) are the key contributors to these processes[7,8]. Both TGF-beta and its receptors are overexpressed in the intestine of CD patients. However, the expression of the TGF-beta isoforms varies with the nature of the tissue. Fibrotic tissue exhibits a reduced expression of TGF-beta 3 and an enhanced expression of TGF-beta 2[9-11]. MMPs are secreted as inactive zymogens which must undergo proteolytic cleavage to become active, and their activity is regulated by specific tissue inhibitors of metalloproteinases (TIMPs)[12-14]. The MMPs do not simply degrade ECM as their name might suggest, but are also responsible for the homeostatic regulation of the ECM. Previous studies have shown that the gene transcription of MMP9 is inducible and that the promoter region is highly responsive to most growth factors and cytokines. They directly cleave and activate growth factors into active ligands, and therefore regulate their bioavailability and/or activity[15-17]. In consequence of these complex interactions of the regulatory processes, the development of the intestinal strictures characteristic of CD cannot be explained simply by the lower or higher expression of one or other of these factors. The key driver of stricture formation rather appears to be an off-balance between the TGF-betas, MMPs and TIMPs which develops in the chronic phase of inflammation. MMP9 is the most abundant MMP expressed in colonic tissue from CD patients, and may therefore be regarded as a biomarker in the evaluation of the clinical activity of inflammatory bowel diseases (IBDs)[18].

The intestinal symptoms common among CD patients are often related to enteric neuropathy. The evidence suggests that both the quantitative properties and function of the myenteric neurons are altered substantially by intestinal inflammation[19-22] and in fact complete loss of the myenteric neurons has been

observed in the strictured regions[23]. However, the extent to which the deficient innervation of the smooth muscle cells (SMCs) and/or the imbalance in the regulation in the molecular events behind the tissue remodelling are responsible for the stricture formation remains unclear.

We recently reported on a rat model of chronic colitis where the mortality was negligible despite the severity of the intestinal symptoms. We demonstrated that experimentally provoked recurring periods of acute inflammation exerted a preconditioning effect against the mucosal damage and reduced the rapid, significant and widespread loss of myenteric neurons observed after the induction of the colitis[24]. In the present work, we used this model to investigate the long-term consequences of acute inflammation on the structural and molecular alterations in the strictured gut wall. The aim of the study was to investigate the possible coincidence between the expressions of TGF-betas, MMP9 and TIMP1 behind the structural remodelling of the strictured gut wall. The structural findings at the light- and electron microscopic levels and the molecular findings at the mRNA and protein levels will be discussed.

MATERIALS AND METHODS

Animal model

All procedures involving experimental animals were approved from the Local Ethics Committee for Animal Research Studies at the University of Szeged. Adult male Sprague-Dawley rats weighing 200-220 g were used throughout the experiments. The animal protocol was designed to minimize pain or discomfort to the animals. The rats were acclimatized to laboratory conditions (23 ℃, 12 h/12 h light/dark, 50% humidity, *ad libitum* access to food and water) for two weeks prior to experimentation. Colitis was induced locally under pentobarbital anaesthesia (45 mg/kg *ip*) by the administration of 2,4,6-trinitrobenzenesulfonic acid (TNBS; Sigma-Aldrich, St. Louis, MO, United States; 10 mg) dissolved in 0.25 mL of 25% ethanol, as described earlier[24]. Repetitive relapsing inflammation (RRI) was mimicked through repeated administration of the same TNBS doses. The rats were treated once (n = 8), twice (n = 7) or three times (n = 8) with TNBS, 2 weeks passed between the treatments. Control rats (n = 18) received an enema of 0.25 mL of 9 g/L saline at the same time as the TNBS was administered. The rats were weighed and monitored daily for activity, bloody diarrhoea and mortality and were sacrificed 60, 90 or 120 d after the last TNBS or saline administrations.

Tissue handling

The animals were killed by cervical dislocation under pentobarbital anaesthesia. After this the last 8 cm region of the descending colon from the anus was dissected. Digital photographs were taken to evaluate the frequency and macroscopic extent of the strictures. Three colonic tissue samples were taken from each animal: the stricture itself and samples adjacent proximally and distally to it. Colonic samples of age-matched controls were also collected. Small pieces (2-3 mm) of the colonic segments for light- and electron microscopic morphometry and post-embedding immunohistochemistry were fixed in 20 g/L formaldehyde and 20 g/L glutaraldehyde solution and embedded in Epon (Electron Microscopy Sciences, Hatfield, PA, United States). Gut segments for molecular studies were cut along the mesentery and pinched flat. After longitudinal cutting, the mucosa and submucosa were removed. Half of the colon samples were immediately frozen in liquid N_2 and later processed for western blot analysis. The other half were incubated overnight at 4 ℃ in RNA Later (Qiagen, Venlo, The Netherlands) and stored at -80 ℃ until processing for quantitative real-time PCR (qRT PCR).

Light- and transmission electronmicroscopic morphometry

The Epon blocks were used to prepare semithin (0.7 μm) sections, which were stained with 10 g/L toluidine blue solution for the light-microscopic study. In the selected area of interest in the semithin cross-sections, all the layers of the gut wall were well oriented. The thicknesses of the LMM and the external circular (CM) and longitudinal (LM) smooth muscle layers were measured at random points with Image J 1.44 (National Institute of Health, Bethesda, MD, United States). The same Epon blocks were used to prepare ultrathin (70 nm) sections and the samples were mounted on nickel grids. Three grids per block were stained with uranyl acetate (Merck, Darmstadt, Germany) and lead citrate (Merck) and were examined and photographed with a Philips CM 10 electronmicroscope equipped with a MEGAVIEW II camera. The width of 15 tight junctions (TJs), *i.e.,* the distance between adjacent enterocytes, was measured at a magnification of × 46000 in the control samples and in the strictures by using the AnalySIS 3.2 program (Soft Imaging System GmbH, Münster, Germany). The distance between SMCs was determined to evaluate the expansion of the ECM within the muscularis externa (ME). Ten montage photographs per intestinal segment were made at a magnification of × 10500 and the distance of SMCs was evaluated in limited-size (2000 nm × 2000 nm) grids for all images, at the intersection of the grid lines, perpendicularly to the cells and calculated by using the AnalySIS 3.2 program. The mean distance was calculated by using the AnalySIS 3.2 program.

Post-embedding immunohistochemistry

The Epon-embedded tissue blocks used previously for the morphometry also served for the post-embedding immunohistochemistry of caspase 9, as described

earlier[25]. Briefly, ultrathin sections from each block were sequentially incubated with anti-caspase 9 (Sigma-Aldrich, St. Louis, MO, United States; final dilution 1:50) primary antibodies overnight, followed by protein A-gold-conjugated anti-rabbit (18 nm gold particles, Jackson ImmunoResearch, West Grove, PA, United States; final dilution 1:20) secondary antibodies for 3 h, with extensive washing between. Sections were counterstained with uranyl acetate and lead citrate, and then examined and photographed with a Philips CM10 electronmicroscope equipped with a MEGAVIEW II camera. The numbers of gold particles were counted on digital photographs at a magnification of × 25000 in 10 SMCs and at a magnification of × 34000 in 5 myenteric ganglia (MGs) per colonic segment in each experimental groups with the AnalySIS 3.2 program.

Statistical analysis

Statistical analysis of the histological results was performed by using one-way ANOVA and the Newman-Keuls test with GraphPad Prism 4.0 (GraphPad Software, La Jolla, CA, United States), and a probability $P < 0.05$ was set as the level of significance. The results were expressed as mean ± SE. The statistical methods of the study were reviewed by Mária Bagyánszki from University of Szeged.

Quantitative real-time polymerase chain reaction

Tissue samples were homogenized in AccuZol (Bioneer, Daejeon, South Korea) directly before qRT PCR. Total RNA was prepared from tissue homogenates as suggested by the manufacturer (Bioneer, Daejeon, Korea). The reverse transcription was achieved by using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, United States) as described earlier[24]. qRT PCR was performed in an Exicycler 96 (Bioneer, Daejeon, Korea) in a total volume of 20 μL containing 10 μL of FastStart SYBR Green PCR Master Mix, 1 μL of specific primer (0.5 pmol/μL) and 50 ng of cDNA template. The PCR program began with a 15-min initial step at 95 ℃, followed by 45 cycles of 15 s at 95 ℃ for denaturation, 45 s at 60 ℃ for annealing and 25 s at 72 ℃ for extension. The sequences of primers were derived from NCBI RefSeq Database entry NM_031131.1 for TGF-beta 2 (forward: 5′ agtgggcagcttttgctc 3′ and reverse: 5′ gtagaaagtgggcgggatg 3′), NM_013174.2 for TGF-beta 3 (forward: 5′ gaagagggccctggacac 3′ and reverse: 5′ gcgcacacagcagttctc 3′), NM_031055.1 for MMP9 (forward: 5′cctctgcatgaagacgacataa 3′ and reverse: 5′ ggtcaggtttagagccacga 3′) and NM_053819.1 for TIMP1 (forward: 5′ cagcaaaaggccttcgtaa 3′ and reverse: 5′ tggctgaacagggaaacact 3′). Hypoxanthine guanine phosphoribosyltransferase (HPRT) (NBCI RefSeq Database entry: NM_012583.2; forward: 5′ gaccggttctgtcatgtcg 3′ and reverse 5′ acctggttcatcatcactaatcac 3′) was used as a housekeeping gene to normalize the expression data. The results were expressed as mean ± SD.

Western blotting analysis and gelatine zymography

Tissue samples were homogenized in TRIS-mannitol buffer and the total cellular protein was then denaturated (mixing and boiling with v/v 20 mmol/L Tris 7-9, 3 mmol/L EDTA, 20 g/L sodium dodecyl sulphate (SDS), 100 g/L mercaptoethanol and 200 g/L glycerol) from each sample as described earlier[26]. Aliquots of 10 μg of total cellular protein were electrophoresed by 100 g/L SDS-polyacrilamide gel, and transferred to nitrocellulose membrane (Amersham, Buckinghamshire, United Kingdom). Two hours after blocking (with PBS pH 7.4, 2.5 g/L Tween 20 (v/v) and 50 g/L non-fat dried milk), the membranes were probed with anti-MMP9 mouse monoclonal antibody (Abcam PLC, Cambridge, United Kingdom; final dilution 1:1000) or TIMP1 (H150) rabbit polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, United States; final dilution 1:1000) for 2 h, and then incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, United States; final dilution 1:2000) for 1 h at room temperature with extensive PBS-Tween 20 washing between. Immunoreaction was visualized with an Immobilon Western HRP Substrate enhanced chemiluminescence system (Millipore Corporation, Billerica, MA, United States) and scanned with a LI-COR C-DiGit™ Blot Scanner (Li-Cor Corporate, Lincoln, NE, United States).

The activity of MMP9 was determined by gelatine zymography, performed by diluting colonic homogenates in zymogram sample buffer (Bio-Rad, Hercules, CA, United States) and electrophoresing the samples in precast 100 g/L SDS-PAGE containing gelatine (20 mg/mL; Sigma-Aldrich, St. Louis, MO, United States) at 120 V until resolution was achieved. The gels were removed from their casings, gently rinsed in ddH_2O, placed onto a shaker in 1X renaturation buffer (Bio-Rad, Hercules, CA, United States) for 40 min, and then placed in 1X development buffer (Bio-Rad Hercules, CA, United States). With change of the buffer once at 20 min, the gels were next incubated at 37 ℃ for 20 h and stained with Coomassie Blue (Bio-Rad, Hercules, CA, United States) for 40 min before being destained in water for 1 h and scanned with a LI-COR C-DiGit™ Blot Scanner.

RESULTS

General observations

Despite the severity of the acute intestinal inflammation of the TNBS-treated rats, the mortality was negligible: only 2 rats died throughout the 120-d experimental period. By 1 d following the TNBS treatment, all the animals had developed symptoms such as weakness, weight loss and bloody diarrhoea. However, by 7 or 8

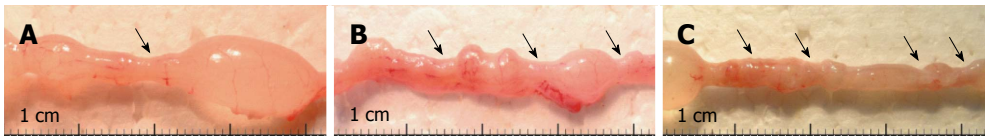

Figure 1 Representative micrographs from the distal colon of rats with chronic colitis 90 d after the first (A), second (B) or third (C) treatment with 2,4,6-trinitrobenzenesulfonic acid. The frequency and size of the strictures (arrows) increased in the time and with the number of 2,4,6-trinitrobenzenesulfonic acid (TNBS) administrations.

d after TNBS administrations, all the visible symptoms accompanied by acute inflammation had resolved. By day 60 following TNBS treatments, all the rats that had previously been exposed to acute colitis had regained their initial body weight and strictures had appeared in each TNBS-treated group. We, therefore, investigated the structural and molecular characteristics of the strictured gut wall from this timepoint on. Whereas the numbers and sizes of the strictures increased in time and with the number of TNBS treatments, they always developed within the previously inflamed colonic areas (Figure 1) and, once they had appeared, their structure and molecular characteristics did not differ. To avoid repetitions therefore, representative results will be presented here, obtained after the processing of tissue samples collected exclusively after the third TNBS administration.

Light microscopy

Representative images of toluidine blue-stained semithin sections of colon where the thickness of the ME was measured are shown in Figure 2. Such colonic sections were collected for measurements on days 90 and 120 following TNBS administrations and also from age-matched controls. The strictured colonic regions displayed normal mucosal architecture and clearly defined, yet thickened muscle layers (not shown). Morphometric analyses revealed the approximately 2-fold thickening of the LMM and layers of the ME in the strictured region relative to the control samples on 90 d. While further significant thickening of the ME was measured beyond day 90 after TNBS administrations, the thickness of the LMM at later than 90 d was similar to that in the controls (Figure 2).

Transmission electronmicroscopy

Transmission electronmicroscopic examination of the colonic epithelium in the strictured region on days 90 and 120 after TNBS administrations showed that the apical surface of the enterocytes with intact brush-border and closed TJs was similar to that in the controls (Figure 3). The width of the TJs between adjacent enterocytes was evaluated morphometrically and was always found to be less than 3 nm (data not shown). However, autophagosome-like double-membrane vesicles of different sizes were frequently seen within the enterocytes (Figure 3).

Because of the excess accumulation of ECM elements in the strictured colonic regions, the SMCs had moved away from each other significantly by day 90 after TNBS administrations, and by 120 d there was more than 2-fold increase in the distance between adjacent SMCs as compared with the controls (Figure 4). Because of the ECM deposition, the SMCs also moved away from the MGs (Figure 4). By day 120 post-TNBS treatments, swollen and empty confluent vacuoles and autophagosomes were frequently seen in the SMCs and also in their close environment, together with different cell organelles in the strictured colonic areas (Figure 4). The vast majority of the axons appeared normal, but necrotic axons were seen rarely in the MGs (Figure 4). Quantitative post-embedding immunohistochemistry in the strictured areas revealed a progressive increase in the number of gold particles indicating caspase 9 antigen in the SMCs and MGs relative to the control samples (Figure 5). The caspase 9-labelling gold particles in the MGs were mainly associated with the mitochondria (Figure 5), the ultrastructure of which was well preserved even 120 d after TNBS treatments.

Quantitative changes in TGF-beta, MMP9 and TIMP1 mRNA and protein expression

On day 90, the TGF-beta 2 mRNA was up-regulated, while the TGF-beta 3 mRNA was down-regulated in the strictured gut wall and also in the colonic segments adjacent proximally and distally to the strictures as compared with the controls. The TGF-beta 2 mRNA expression progressively increased, while the TGF-beta 3 mRNA expression further decreased by day 120 in all three segments (Figure 6A).

A marked overexpression of MMP9 mRNA was detected in all the colonic segments examined on days 90 and 120 after TNBS treatments (Figure 6B). At the same timepoints, the TIMP1 mRNA expression was up-regulated in the colonic segments adjacent proximally and distally to the strictures, but was down-regulated in the strictures themselves (Figure 6B). MMP9 and TIMP1 expression was also evaluated at the protein levels. Although a high amount of MMP9 protein was demonstrated in the tissue samples from the control rats, the progressive increase in the amount of MMP9 protein in the strictures was obvious between days 90 and 120 (Figure 6C). Gelatine zymography demonstrated that an active form of MMP9 protein rather than pro-MMP was expressed (Figure 6C). While the amount of TIMP1 protein also decreased acutely between days 90 and 120 in the control samples, it was

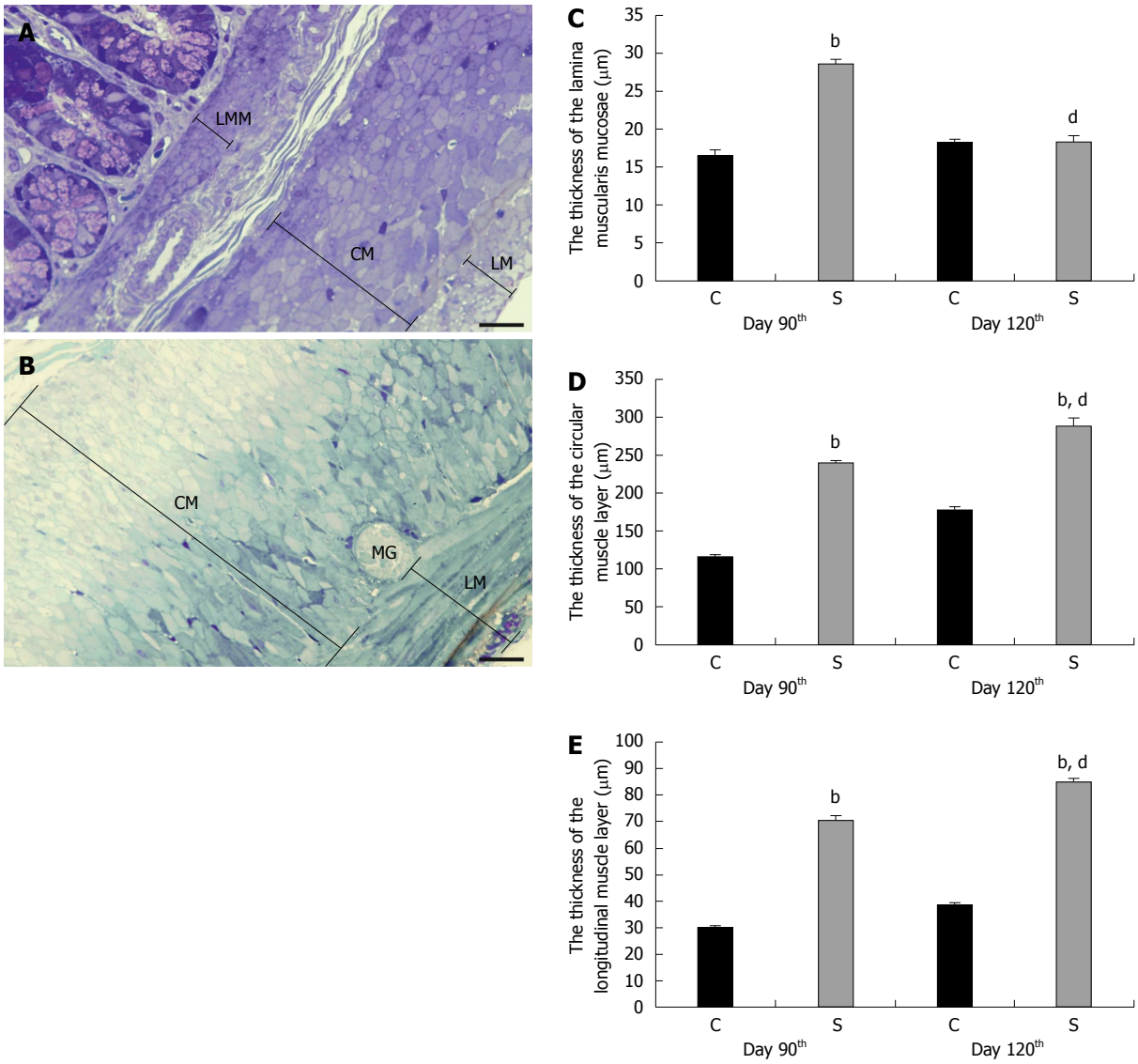

Figure 2 Thickness of the smooth muscle layers in the colon of control animals and in rats treated three times with 2,4,6-trinitrobenzenesulfonic acid. A: Representative light micrographs of a toluidine blue-stained semithin section from the colon of a control rat; B: TNBS-treated rat on day 120 of the experimental period. Bar: 25 μm. Significant thickening of the LMM (C), CM (D) and LM (E) was demonstrated in the strictured gut wall of the TNBS-treated rats (S) relative to the controls (C) on day 90. Whereas a further significant thickening was measured in the CM and LM on day 120, the thickness of the LMM was similar to that in the controls at this timepoint. Data are expressed as mean ± SE. $^{b}P < 0.001$ TNBS-treated groups *vs* age-matched controls; $^{d}P < 0.001$ 2,4,6-trinitrobenzenesulfonic acid (TNBS)-treated group on day 90 *vs* TNBS-treated group on day 120. LMM: Lamina muscularis mucosae; CM: Circular muscle layer; LM: Longitudinal muscle layer; MG: Myenteric ganglion.

practically undetectable in the strictures (Figure 6C).

DISCUSSION

We recently reported on a rat model in which alleviated inflammatory damage in association with the persistent up-regulation of HO-1 were salient features in the acute phase of intestinal inflammation induced by repeated TNBS administrations[24]. The same model was used in the present work to investigate the structural and molecular events leading to the formation of a strictured gut wall. Concerning the long-term consequences of the acute inflammation in this model, all the visible symptoms had resolved by day 60 after TNBS administration, the body weight of the treated rats was similar to that of the age-matched controls, and intestinal strictures developed in all of the rats that had previously displayed intestinal inflammation. These findings accord well with the clinical observations that mucosal healing and clinical remission alone cannot be treatment endpoints in CD, because this does not prevent later stricturing[27,28]. The increases in size and frequency of the strictures observed here after 60 d provide experimental evidence in favour of the view that strictures, once present, gradually progress and, once fibrosis develops, it cannot be reversed[29].

Aligned thickening of all the muscle layers in the strictured gut wall until up to day 90 after TNBS administrations was characteristic. Whereas the thic-

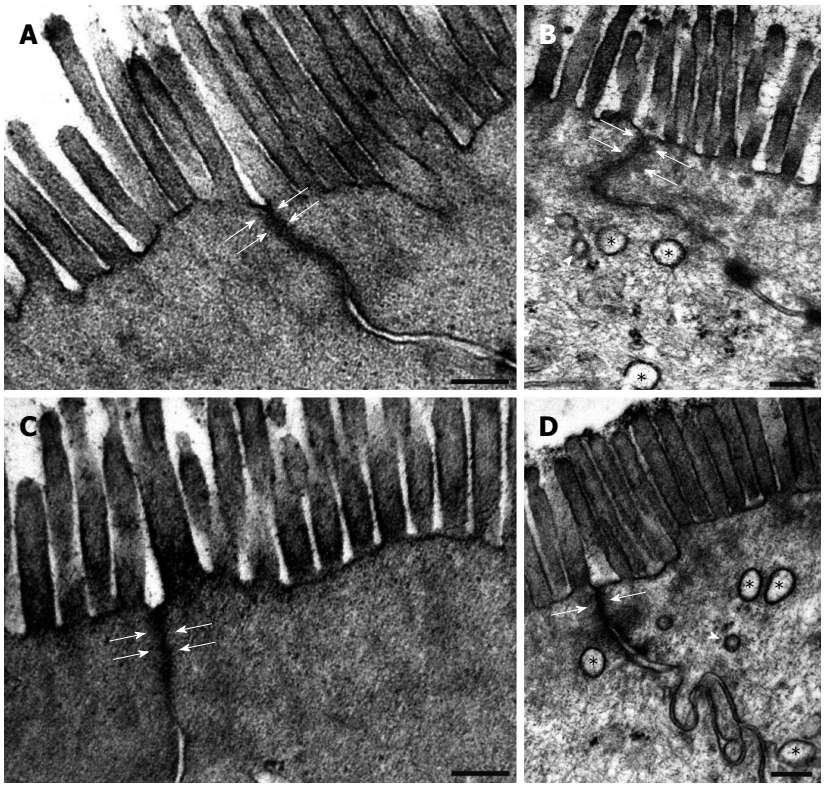

Figure 3 Representative electron micrographs of two neighbouring enterocytes from the colon of control animals (A, C) and in rats treated three times with 2,4,6-trinitrobenzenesulfonic acid. On days 90 (B) and 120 (D) following the third 2,4,6-trinitrobenzenesulfonic acid (TNBS) administration, both the microvillar surface and the width of the apical intercellular tight junctions (arrows) of the enterocytes in the strictured regions were similar to those in the age-matched controls (A on day 90 and C on day 120). However, autophagosomes (asterisks) and lysosomes (arrowheads) were commonly observed within the epithelial cells of the strictured gut wall. Bars: 200 nm.

kening of the ME progressed further and became a decisive element of the strictured gut wall, the thickness of the LMM did not change after day 90, and did not differ from that in the controls by the end of the experimental period. Since the LMM is most involved in maintaining the mucosal integrity[30], we suppose that the earlier cessation of excess ECM deposition in the LMM is a consequence of the differential regulation of inflammation-related events here through cytokines derived from the epithelium[31,32].

At 90 and 120 d following TNBS administrations, transmission electronmicroscopy showed that the structures of the epithelium necessary to maintain the barrier functions were intact. However, the frequent presence of double-membrane autophagosomes indicated high levels of intracellular stressors in the previously affected epithelium. It has been well documented that induction of autophagy is a determining factor for the maintenance of cellular homeostasis in chronic colitis[33,34]. The importance of autophagy in the pathogenesis of chronic intestinal inflammation has also been demonstrated by genome-wide association studies which indentified a link between the genes involved in autophagy regulation and IBDs[35,36].

While the rapid and widespread loss of myenteric neurons was a characteristic feature of the onset of acute inflammation[24], the precise timing of the cellular events in the chronic phase leading to the intestinal stricturing here showed that the SMCs in the ME were affected first in these processes. Since the excess deposition of ECM in the ME was sustained throughout the experimental period, the SMCs progressively moved away from each other and also from the MGs, leading eventually to deficient innervation and severe cellular damage. After day 60 following TNBS treatments, the appearance of autophagosomes, the leakage of cellular contents and the increasing number of gold particles labelling caspase 9 expression indicated that all three types of cell death mechanisms had already progressed in the SMCs by day 90 when necrotic axons were only rarely seen in the MGs. As the pathological environment became more extensive with time, by day 120 after TNBS administration locally severe neuronal injury also occurred in the strictured tissue as a significant sign of chronic inflammation, similarly as described in other models[23,37]. As regards the timing of the events, we presume suppose that the neuronal injury is a consequence and not the cause of the stricturing processes.

Evidence from both animal models[38,39] and human studies[18,40,41] has suggested that the up-regulation of TGF-beta 2 and of MMP9 may be considered to be biomarkers in the post-inflammatory tissue

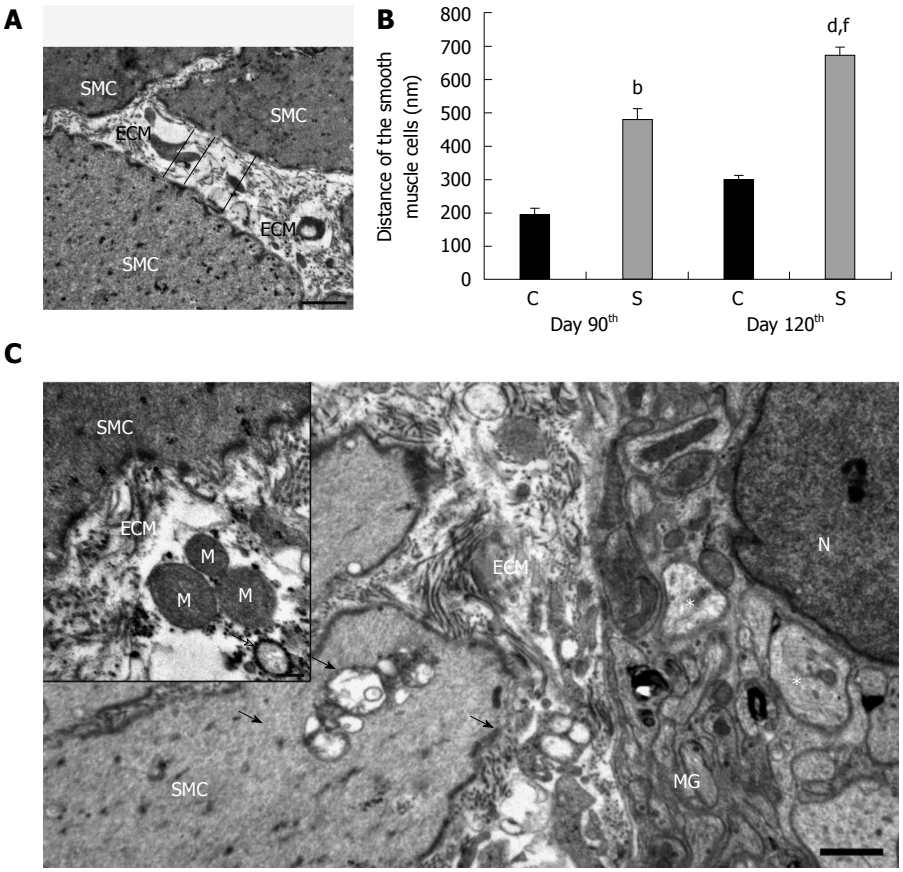

Figure 4 Ultrastructural alterations within the colon of control animals and in rats treated three times with 2,4,6-trinitrobenzenesulfonic acid. Excess deposition of extracellular matrix (ECM) was observed within the smooth muscle layers in the ultrathin sections derived from the strictured region (A); Electronmicroscopic morphometry revealed that the distance between adjacent smooth muscle cells (SMCs) was significant larger in the strictured gut wall of the 2,4,6-trinitrobenzenesulfonic acid (TNBS)-treated rats (S) as compared with the gut wall of the control rats (C) on day 90 (B); A further significant increase in the mean separation distance of the SMCs was recorded on day 120 post-TNBS treatment (B). Data are expressed as mean ± SE. $^{b}P < 0.01$, $^{d}P < 0.001$ TNBS-treated groups *vs* age-matched controls; $^{f}P < 0.001$ TNBS-treated group on the day 90 *vs* TNBS-treated group on day 120. Representative electron micrograph of the strictured colonic area 120 d after the third TNBS administration (C). Because of ECM accumulation, the SMCs also moved away from the myenteric ganglia (MGs). Swollen and empty confluent vacuoles of different sizes (arrows) were frequently seen in the SMCs and also in their close environment. Rupture of the plasma membrane and subsequent leakage of the cell organelles into the microenvironment, *e.g.*, the mitochondria (M) and autophagosomes (hollow arrow), were frequently seen in the intercellular spaces (insert). However, the vast majority of the axons appeared normal; necrotic axons were rarely seen in the MGs (asterisks). N: Nucleus. Bars: 1 μm and 200 nm (insert).

remodelling leading to stricturing in CD. The mRNA expression profile of the TGF-beta isoforms, the up-regulation of TGF-beta 2 and the down-regulation of TGF beta 3 in the colonic segments examined in our model accorded well with the distinctive expressional profile of the secreted TGF-beta isoforms in human CD primary intestinal myofibroblasts[41]. The spreading of this characteristic expression pattern both proximally and distally to the strictures indicated the bidirectional diffusion of the disease along the colon in our model. We also detected progressive up-regulation of MMP9 mRNA in all three colonic segments, suggesting again the proximally and distally directed diffusion of the pathological environment. However, the MMP9 up-regulation in the strictured gut wall was coupled with the down-regulation of TIMP1, and an increased amount of active MMP9, but no TIMP1 protein was detected here, indicating a stricture-specific off-balance in the production of proteases and their inhibitors. This expression pattern is very reminiscent of that which develops in the fistulae in approximately one-third of patients with CD[42]. The apparent differences in expression profiles between our study and the literature data in tissue samples prepared from the control guts could be explained by the different methodological approaches. The novelty of our studies was that we prepared tissue homogenates for molecular studies not from the mucosa overlying the strictures, but exclusively from the ME, where the background events of the chronic inflammation leading to stricture formation actually occurred.

In conclusion, The structural and molecular events leading to stricturing as a long-term consequence of acute intestinal inflammation that were demonstrated earlier in animal models and in human studies also characterized the stricture formation induced in our rat model by repeated TNBS administrations. Since the exact timing of the stricturing processes was possible in this model, we reached the conclusion that, in contrast with the general view, the ME, and not the

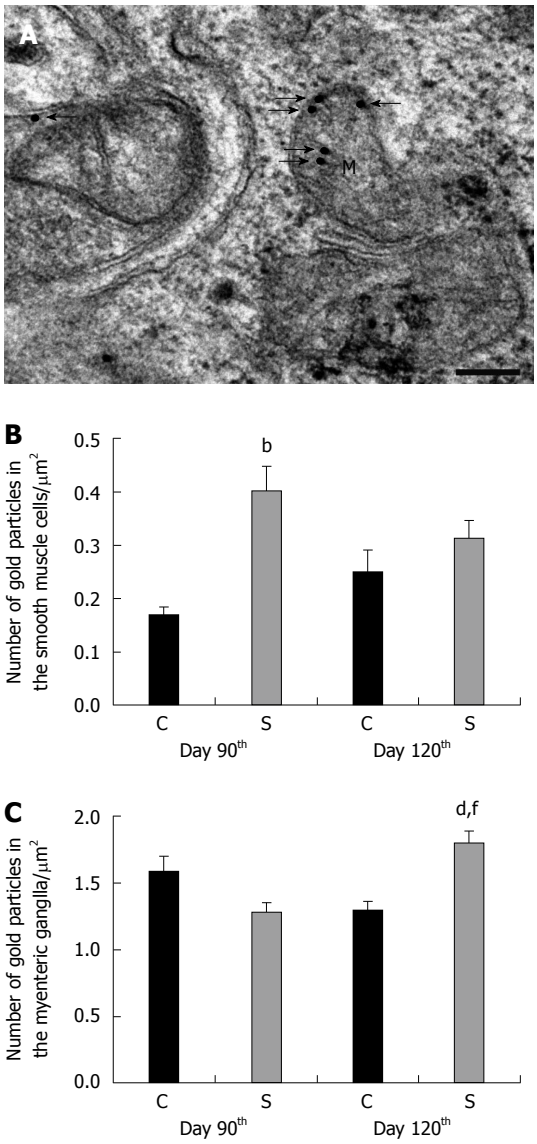

Figure 5 Post-embedding immunogold labelling for caspase 9 in the smooth muscle cells and myenteric ganglia in the colon of control animals and in rats treated three times with 2,4,6-trinitrobenzenesulfonic acid. Representative electron micrograph of a myenteric ganglion (MG) from the strictured gut wall (S) 120 d after the third 2,4,6-trinitrobenzenesulfonic acid (TNBS) administration (A). The 18 nm gold particles labelling caspase 9 immunoreactivity (arrows) were mainly associated with mitochondria (M). Bar: 200 nm. The number of gold particles in the S was increased significantly in the smooth muscle cells on day 90 (B) and also in the MGs on day 120 (C) as compared with the gut wall in the control rats (C). Data are expressed as mean ± SE. $^{b}P < 0.01$, $^{d}P < 0.001$ TNBS-treated groups *vs* age-matched controls; $^{f}P < 0.01$ TNBS-treated group on the day 90 *vs* TNBS-treated group on day 120.

epithelial barrier or the MGs, was the primary target of the events leading to stricture formation. Moreover, this TNBS-induced rat model has provided the first experimental demonstration of the molecular diffusion of the disease both proximally and distally along the gut wall. The off-balance in MMP9/TIMP1 expression profile found strictly within the border of the strictures may well allow use of this model to investigate the molecular mechanisms leading to fistulated CD.

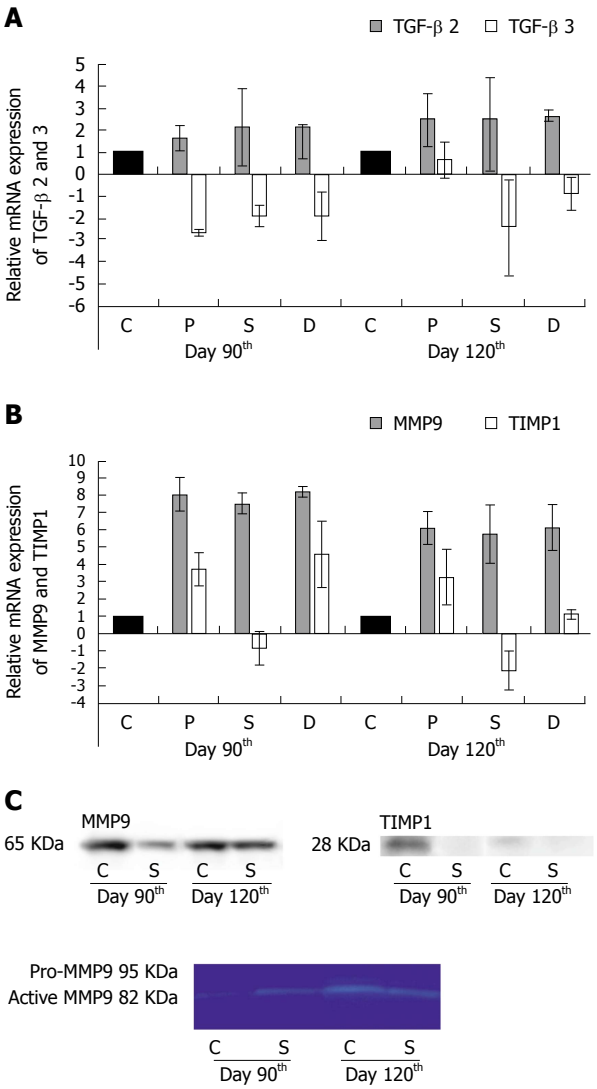

Figure 6 Relative mRNA and protein expression of transforming growth factor-beta 2 and 3, matrix metalloproteinase 9 and tissue inhibitor of metalloproteinases 1 in the colon of control animals and in rats treated three times with 2,4,6-trinitrobenzenesulfonic acid. TGF-beta 2 was up-regulated in the strictured region (S) and also in the adjacent proximal (P) and distal (D) segments of the colon as compared with the controls (C) on day 90 and day 120 (A). TGF-beta 3 gene repression was detected both 90 and 120 d after the third TNBS treatment in each colonic segment (A). The marked overexpression of MMP9 mRNA was confirmed in each colonic segment in the chronic phase of the inflammation (B). TIMP1 mRNA expression was detected in the P and D colon segments at both timepoints examined, but in the S the gene was down-regulated (B). Data are expressed as mean ± SD. 90 d after the third TNBS treatment, a decreased MMP9 protein level was detected in the S relative to the C (C, upper). Nevertheless, on day 120 the MMP9 protein expression was similar to that in the C. The activity of MMP9 was determined by gelatine zymography (C, lower). An active form of the MMP9 protein rather than pro-MMP9 was expressed in the C and S segments at both timepoints examined. Well-detectable amounts of TIMP1 protein were revealed only in the control samples from day 90 (C, right side).

COMMENTS

Background

Intestinal strictures are characteristic complications of Crohn's disease (CD) affecting more than one third of all patients. Its can lead to partial or total

intestinal obstruction with potentially life-threatening consequences. Although the treatment of the chronic complications of CD is a serious medical problem, the pathogenesis, factors, and cell types involved in stricture formation are largely unknown.

Research frontiers

Despite of the huge amount of animal models and human studies, the structural and molecular events leading to stricturing as a long-term consequence of acute intestinal inflammation are still not clear until today. Besides, the ultrastructure of the intestinal strictures is still unknown.

Innovations and breakthroughs

This TNBS-induced rat model has provided the first experimental demonstration of that, in contrast with the general view, the muscularis externa, and not the epithelial barrier or the myenteric ganglia, was the primary target of the events leading to stricture formation.

Applications

The authors hypothesize form the results derived our rat model with chronic colitis and very low mortality that the experimentally provoked recurrent relapsing inflammations characteristic to CD can provoke the recrudescence of the strictures post-surgically despite of the complete mucosal healing and restoring myenteric neuronal injury.

Terminology

The authors described earlier that experimentally provoked repetitive relapsing inflammations develop preconditioning effect by speeding up mucosal healing and restoring myenteric neuronal injury.

Peer-review

This is a well-written manuscript with carefully designed and described experiments. The observations are interesting and certainly add to our knowledge in the inflammation-induced fibrosis in the large intestine.

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ORIGINAL ARTICLE

Basic Study

Apoptosis induced by a low-carbohydrate and high-protein diet in rat livers

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Institutional review board statement: The study was authorized and approved by the Director of Nutrition College of Fluminense Federal University and by the professor responsible for the Experimental Nutrition Laboratory of the same institution.

Institutional animal care and use committee statement: The study received prior approval by the Institutional Review Board for Animal Research (CEUA), Fluminense Federal University, case number 648, February 27, 2015. It was designed based on the determinations of the Brazilian law for research with animals (law number 11.794, October 2008).

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Abstract

AIM: To determine whether high-protein, high-fat, and low-carbohydrate diets can cause lesions in rat livers.

METHODS: We randomly divided 20 female Wistar rats into a control diet group and an experimental diet group. Animals in the control group received an AIN-93M diet, and animals in the experimental group received an Atkins-based diet (59.46% protein, 31.77% fat, and 8.77% carbohydrate). After 8 wk, the rats were anesthetized and exsanguinated for transaminases analysis, and their livers were removed for flow cytometry, immunohistochemistry, and light microscopy studies. We expressed the data as mean ± standard deviation (SD) assuming unpaired and parametric data; we analyzed differences using the Student's *t*-test. Statistical significance was set at $P < 0.05$.

RESULTS: We found that plasma alanine aminotransferase and aspartate aminotransferase levels were significantly higher in the experimental group than in the control group. According to flow cytometry, the percentages of nonviable cells were 11.67% ± 1.12% for early apoptosis, 12.07% ± 1.11% for late apoptosis, and 7.11% ± 0.44% for non-apoptotic death in the experimental diet group and 3.73% ± 0.50% for early apoptosis, 5.67% ± 0.72% for late apoptosis, and 3.82% ± 0.28% for non-apoptotic death in the control diet group. The mean percentage of early apoptosis was higher in the experimental diet group than in the control diet group. Immunohistochemistry for autophagy was negative in both groups. Sinusoidal dilation around the central vein and small hepatocytes was only observed in the experimental diet group, and fibrosis was not identified by hematoxylin-eosin or Trichrome Masson staining in either group.

CONCLUSION: Eight weeks of an experimental diet resulted in cellular and histopathological lesions in rat livers. Apoptosis was our principal finding; elevated plasma transaminases demonstrate hepatic lesions.

Key words: Apoptosis; Liver injury; High-protein diet; High-fat diet; Low-carbohydrate diet

Core tip: Obesity is a serious and growing health problem. A high-protein, high-fat, and low-carbohydrate diet known as the Atkins diet has been adopted since the 1970s. Many people adhere to this diet in an attempt to lose weight, and it has recently been introduced for children with difficult-to-control seizures and elderly suffering from Alzheimer's and Parkinson's diseases. The benefits and effects of the Atkins diet remain unclear, especially in hepatic metabolism. Since the primary metabolic reactions involving macronutrients occur in the liver, it is essential to understand the potential hepatic lesions that can result from dietary modifications.

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INTRODUCTION

The prevalence of obesity is increasing worldwide among adults, youth, and children; this serious health problem requires widespread public mobilization in the search for solutions. Extremely high numbers of people adhere to special diets in an attempt to lose weight[1]. A high-protein, high-fat, and low-carbohydrate diet was announced by the American cardiologist Robert C. Atkins in the mid-1970s as the best and healthiest way to become slim[2]. This so-called "Atkins diet" or "protein diet" has continued to be viewed as "a matter of love or hate"[3]. This diet was recently introduced for children with difficult-to-control seizures and elderly with Alzheimer's and Parkinson's diseases[4]. Despite numerous medical publications related to the Atkins diet, the results from a majority of such studies are inconclusive and have failed to demonstrate benefits and effects, especially in hepatic metabolism[5].

Several studies have reported associations between a high-protein diet and alterations in the liver[6], intestinal mucosa[7], kidneys[8,9], pancreas[10], adipose tissue[11], and bones[9,12]. Since the primary metabolic reactions involving macronutrients occur in the liver, it is essential to understand the potential hepatic lesions that may result from dietary modifications. It has been shown that high-fat or low-carbohydrate diets can cause hepatic steatosis related to excessive demand for fatty acids from diet and from adipose tissues as a consequence of gluconeogenesis[5]. In a recently published study, a high-protein diet (independent of the amount and type of fat or carbohydrate) was found not to lead to steatosis and may actually reverse it[13].

Hepatic cells are important targets for lesions in the presence of excessive dietary components since they are absorbed through the intestinal mucosa and quickly reach the liver through the portal vein[6]. Among macronutrients, carbohydrates and fat are largely responsible for the observed alterations since they promote changes in gene transcription and glycolytic and lipogenic enzymes [sterol responsive binding protein 1/2 (SREBP) and the mammalian target of rapamycin - mTOR), insulin, and adipokines][14].

Hepatocytes respond to injuries *via* various mechanisms, of which the most important are autophagy, apoptosis, and non-apoptotic death[15]. Autophagy may be considered to be an adaptive process associated with different types of liver injury, such as nutrient deprivation, insufficient growth factor, hypoxia, and the accumulation of fat in hepatocytes. Autophagy can be reversed if conditions improve. The cell digests its own components for use as an energy substrate; and when these components are insufficient to maintain cell homeostasis, either apoptosis or non-apoptotic death occurs[16,17]. Apoptosis, a cellular suicide program, is a natural process that is necessary to remove damaged, senescent, or mutagenic cells that have completed their mission. However, this process may lead to the development of various liver diseases. It is responsible for the development of nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) when the capacity of hepatocytes to store free fatty acids from the diet is exceeded[18]. Apoptosis is an active process that requires energy; when energy is insufficient, the process decelerates and non-apoptotic death begins. Non-apoptotic death differs from apoptosis in many ways since it sparks an

inflammatory response that can aggravate the initial lesion[15,18].

Additional histopathological studies of livers of animals fed a low-carbohydrate and high-protein diet are required since the results to date have been conflicting[19,20].

Here, we hypothesized that a high-protein (59.46%), high-fat (31.77%), and very low-carbohydrate (8.77%) diet can cause hepatocyte lesions, as identified by flow cytometry and immunohistochemistry (IHC). We aimed to correlate the cytometry findings with light microscopy alterations and plasma transaminase levels.

MATERIALS AND METHODS

Animals

The experimental study was performed from March-May 2015 in the Experimental Nutrition Laboratory of the Nutrition College, Fluminense Federal University, Niterói, RJ, Brazil. The animal protocol minimized pain and discomfort to the rats. The experiment used 20 female Wistar rats (*Rattus norvegicus*) ranging in age from 11-13 wk. The animals weighed 211-249 g and were reared at the Laboratory Animal Facility of the Oswaldo Cruz Foundation, Ministry of Health, Rio de Janeiro, Brazil. The animals were kept in group cages with four animals each, for adaptation, over the course of 5 d, receiving water and laboratory diet *ad libitum*. After this period, the rats were separated randomly into two groups of 10 animals each [the control diet group (CDG) and the experimental diet group (EDG)] and individually housed in polypropylene cages with controlled temperature (24 ± 2 ℃) and humidity (60% ± 10%) and an alternating light-dark cycle consisting of 12 h of lightness and darkness.

Diets

The CDG diet consisting of the AIN 93M diet[21] was formulated for the maintenance of adult rats by the American Institute of Nutrition in 1993; we based the elaborated EDG diet on the Atkins diet. Both groups received water and an *ad libitum* diet for 8 wk. The diets were prepared by Pragsoluções Biociências Comércio e Serviços, LTD, Jaú, São Paulo, Brazil. The control diet had the following composition: carbohydrate (76.98%), protein (13.56%), and fat (9.46%). The experimental diet was composed of carbohydrate (8.77%), protein (59.46%), and fat (31.77%). The amount of vitamins, minerals, L-cysteine, choline, and fiber were the same in the two groups, and tert-butylhydroquinone was calculated as 0.002 mg per gram of fat, all based on AIN 93M determinations (Tables 1 and 2).

Experimental procedures and sample collection

On the morning of the day of sacrifice, all of the animals underwent vaginal smears to determine their estrous cycle phase. Animals in estrus were separated and given no more access to food. After 8 h of fasting, the animals were anesthetized *via* an intraperitoneal injection of a solution containing 11.50 mg/100 g body mass of ketamine and 0.10 mg/100 g body mass of xylazine and were exsanguinated by cardiac puncture[22]. They were then sacrificed one at a time, alternating between the experimental and control group. The blood was placed in a heparinized tube and centrifuged for 20 min at 314 rad/s, and the plasma was separated and stored at -80 ℃ until analysis. The liver was removed after withdrawing the blood, and six liver fragments measuring 1 cm^3 each were washed gently with NaCl 0.9%, submerged in a recipient with the same solution and stored in the freezer at -4 ℃ for 2 h prior to performing flow cytometry.

Table 1 Composition of control diet (AIN-93M)

Ingredients	g/100 g	CH (g)	PTN (g)	LIP (g)	FI (g)
Cornstarch	46.5	39.52			
L-cysteine	0.18		0.18		
Choline bitartrate	0.25		0.25		
Mineral mix	3.50	0.77			
Vitamin mix	1	0.97			
Tert-butylhydroquinone	0.008				
Fiber	5				5
Soybean oil	4			4	
Casein (> 85% Protein)	14		11.06		
Sucrose	10	10.00			
Dextrinized cornstarch	15.5	13.95			
Kcal (%)	338.8	260.84	45.96	32	
Macronutrients (%)	100	76.98	13.56	9.46	

The control diet group was fed this diet for 8 wk, elaborated by the American Institute of Nutrition in 1993 for laboratory rodents, with adequate percentages of macronutrient carbohydrate (CH), protein (PTN) and Lipids (LIP), vitamins, minerals and fiber (FI).

Table 2 Composition of experimental diet (based on the Atkins diet)

Ingredient	(g/100 g)	CH (g)	PTN (g)	LIP (g)	FI(g)
Agar	2				2
L-cysteine	0.18		0.18		
Choline bitartrate	0.25		0.25		
Mineral mix	3.5	0.77			
Vitamin mix	1	0.97			
Tert-butylhydroquinone	0.028				
Fiber	5				5
Sucrose	6	6			
Casein(> 85% protein)	20		16		
Powdered chicken breast	60		36	12	
Soybean oil	2			2	
Kcal (%)	352.68	30.96	209.72	112	
Macronutrients (%)	100	8.77	59.46	31.77	

The experimental diet group was fed this diet for 8 wk, elaborated by the authors based on the Atkins diet. The percentage of macronutrients was 2:1 carbohydrate (CH) + protein (PTN)/lipids (LIP) with fiber (FI). The others ingredients had the same amount as recommended for the AIN-93M.

Analytical methods

We measured plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels using

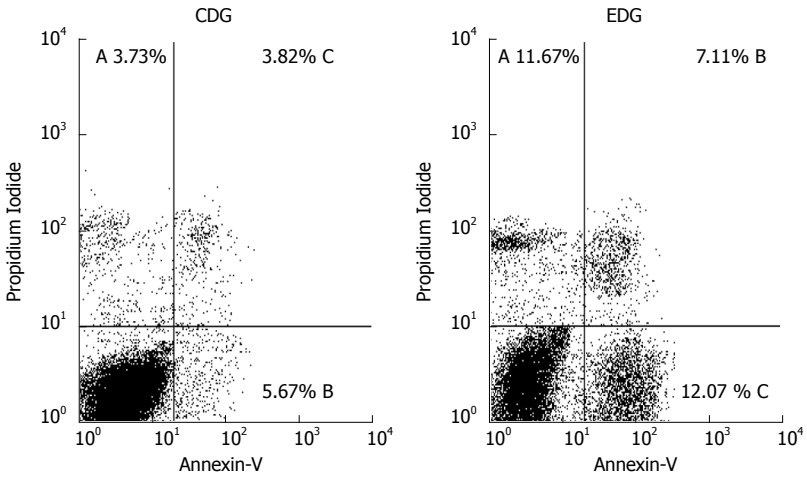

Figure 1 Flow cytometry in hepatocytes from the experimental diet group and control diet group at 8 wk. Annexin-V was used to identify late apoptosis and non-apoptotic death and propidium iodide for early apoptosis. A-% early apoptosis; B-% non-apoptotic death; C-% late apoptosis; D-% viable cells. EDG: Experimental diet group; CDG: Control diet group.

automatic analysis (Vitalab Selectra E, Vital Scientific, Spankaren, Netherlands) with commercial kits from BioSystems Reagents and Instruments (Barcelona, Spain) located in the Multidisciplinary Research Support Laboratory (LAMAP), School of Medicine, UFF, Niterói, RJ, Brazil. The flow cytometry used the following fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit I components: 10X Annexin V Binding Buffer; FITC Annexin V; propidium iodide solution from BD Pharmingen (San Diego, CA, United States). The flow cytometer was the FACF-Calibur BD model. The liver fragments were fixed in Bouin's solution, processed in graded alcohols and xylene, embedded in paraffin blocks, stained for optical microscopy [hematoxylin-eosin (HE) and Trichrome Masson (TM) stains], and prepared for IHC. Alexa fluor 647 rat anti-mouse blimp-1 from BD Pharmingen was used for identifying autophagy by IHC. We performed flow cytometry and IHC in the Biomedical Science Institute of the Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil. We relied on a Zeiss (Oberkochen, Germany) Axioscop 20 microscope and Canon (Tokyo, Japan) G10 camera JPG with 14.7 megapixels at the Pathology Department of the School of Medicine, UFF, Niterói, RJ, Brazil for the optical microscopy.

Statistical analysis

The results of this study are presented using descriptive statistics, such as arithmetic mean and standard deviation. The two-tailed unpaired Student' *t* test was used to compare means between the two groups. It was considered that 95% confidence interval contains the true difference between the means ($P < 0.05$). An *F* test was performed to prove that the data came from two groups that have identical standard deviations (and thus identical variances). The computer program used was Graphpad-Prism version 6.0e (La Jolla, CA, United States) for Mac OS X, 2015, and WinMDI 2.9 was used for flow cytometry. The statistical analyses were reviewed by a biomedical statistician. The IHC and optical microscopy were based on observational evaluation by an expert.

RESULTS

Plasma transaminases

Plasma ALT and AST levels were significantly higher in the EDG than in the CDG. The mean ALT in the EDG was 61.70 ± 4.16 U/L compared with 28.10 ± 4.06 U/L in the CDG ($P < 0.0001$). AST in the EDG was 238.30 ± 15.85 U/L and 179.20 ± 13.86 U/L in the CDG ($P = 0.0117$).

Flow cytometry

Flow cytometry revealed a significantly higher percentage of nonviable cells in the EDG compared with the CDG (30.85% ± 2.20% and 13.22% ± 1.43%, respectively; $P < 0.0001$).

In the EDG, the percentages of nonviable cells were 11.67% ± 1.12% for early apoptosis, 12.07% ± 1.11% for late apoptosis, and 7.11% ± 0.44% for non-apoptotic death. In the CDG, the comparable values were 3.73% ± 0.50% for early apoptosis, 5.67% ± 0.72% for late apoptosis, and 3.82% ± 0.28% for non-apoptotic death (Figure 1).

When comparing the nonviable cells in the two groups, only the mean percentage of early apoptosis was statistically significant (Table 3).

Considering only non-apoptotic death and total apoptosis (early + late), the EDG demonstrated 23.99% ± 2.12% non-apoptotic death and 76.01% ± 2.12% total apoptosis ($P < 0.0001$); the CDG exhibited 29.20% ± 1.29% non-apoptotic death and 70.80% ± 1.29% total apoptosis ($P < 0.0001$).

Immunohistochemistry

IHC was negative for autophagy in both groups.

Table 3 Nonviable cells in experimental diet group and control diet group

	Early apoptosis	Late apoptosis	Non-apoptoic death
EDG[1]	37.34% ± 1.30%	38.67% ± 1.73%	24.00% ± 2.12%
CDG[1]	28.43% ± 1.19%	42.37% ± 1.10%	29.20% ± 1.29%
P-value	< 0.0001	0.0882	0.0512

[1]Each value expressed as mean ± SD (n = 10) significance $P < 0.05$. EDG: Experimental diet group; CDG: Control diet group.

Optical microscopy

Upon examining the livers of the rats in the EDG, the pathologist identified marked sinusoidal dilation around the central vein, with smaller perisinusoidal hepatocytes compared with rats in the CDG, which showed no alteration. The central vein was normal in both groups. Five animals in the CDG had isolated periportal cytoplasmic microvesicles compared with three animals in the EDG. Small and heterogeneously distributed structures were found in the liver of all animals in both groups; these structures likely corresponded to deposition of glycogen. In the EDG, these structures decreased and even absent in some zones. The pathologist observed acute and chronic inflammatory periportal cells in both groups, which are considered to be normal in rats. Fibrosis was not identified by HE or TM in either group (Figure 2).

DISCUSSION

As expected, we found that a high-protein, high-fat, and low-carbohydrate diet caused cellular and histopathological lesions in the livers of experimental rodents. ALT and AST were increased in the EDG compared with the CDG. Since these tests are considered to be precise liver function tests, our results confirmed the presence of liver damage involving hepatocyte destruction with plasmatic membrane disruption and late-phase apoptosis and non-apoptotic death in the EDG[23,24].

In a study by Jean *et al*[25], a group of animals that received a diet consisting of 50% protein exhibited high ALT and normal AST compared with controls that received a modified AIN-93M diet. The results were interpreted as hepatic lesions since ATL is a specific liver enzyme located in the hepatocyte cytoplasm; AST can also be expressed by muscles and kidneys[23,25]. Oarada *et al*[6] demonstrated that when rats were fed increasing amounts of protein (35%, 40%, 45%, and 50%), ALT and AST increased to the same degree. These authors accordingly concluded that protein-independent of other macronutrients and energy consumption-was a risk factor for liver injury. In a recent study, Kostogrys *et al*[26] found no changes in plasma transaminase levels with a diet of 50.0% protein, 37.7% fat, and 12.3% carbohydrate, although the liver was enlarged compared to animals receiving the AIN93-M diet. Comparing our results with those noted in the literature may be difficult since the percentages of macronutrients fed to the rats vary from one study to another.

Flow cytometry confirmed the hepatic damage, as demonstrated by increased plasma transaminase levels; 30.85% of the hepatocytes were nonviable in the EDG compared with 13.22% in control animals. Nonviable cells in the CDG included 3.73% early apoptosis, 5.67% late apoptosis, and 3.82% non-apoptotic death. These findings in the control group can be considered to be physiological since apoptosis and non-apoptotic death represent a continuous process that is responsible for maintaining the balance between proliferation and cellular death. Non-apoptotic death is part of the same process since it is the ultimate fate of cells that undergo apoptosis[24].

Apoptosis was markedly increased in the EDG, with 11.67% early apoptosis, 12.07% late apoptosis, and 7.11% non-apoptotic cells exhibiting a non-physiological state. Any dysregulation of apoptosis is deleterious and results in tissue damage[15]. Similar results were found by Chiang *et al*[27] who demonstrated that mice receiving an 8-wk diet with 60% protein exhibited changes in bodyweight, liver histology, and expression of apoptosis and fibrosis.

Another important finding of the present study was that the percentage of early apoptosis, degeneration of mRNA, was significantly higher in the EDG (37.34%) compared with that in the CDG (28.43%) ($P < 0.0001$), indicating that apoptosis was progressing in the liver[18]. This finding might be evidence that the rats did not adapt to the experimental diet over the 8 wk of the study.

IHC was negative for autophagy in both groups. Autophagy was likely not found in this study because the percentage of nonviable cells increased (*i.e.*, the cytoprotective mechanism was probably suppressed), and apoptosis continued to be active since early apoptosis was higher in the experimental group than in the control group. The two pathways are controlled by common mechanisms: when autophagy is inhibited, apoptosis is induced[16-18]. For comparison, we note that Garbow *et al*[5] fed animals a similar diet and found autophagy among others alterations.

A histological examination of the rats' livers in the EDG revealed sinusoidal dilation around the central vein with smaller perisinusoidal cells compared to the livers of control animals. Similarly Bollo *et al*[28] found small hepatocytes around the central vein in alpine chamois during winter and considered this change an adaptation to under-nutrition. Bollo *et al*[28] asserted that the central vein region was a metabolic zone from which nutrients were distributed to the rest of the organ. The hepatocytes atrophied, and the size of the core was reduced. This probable adaptation to inadequate nutrition could be considered a strategy to minimize energy expenditures.

Cytoplasmic microvesicles were observed in five rats fed the control diet and three rats fed the

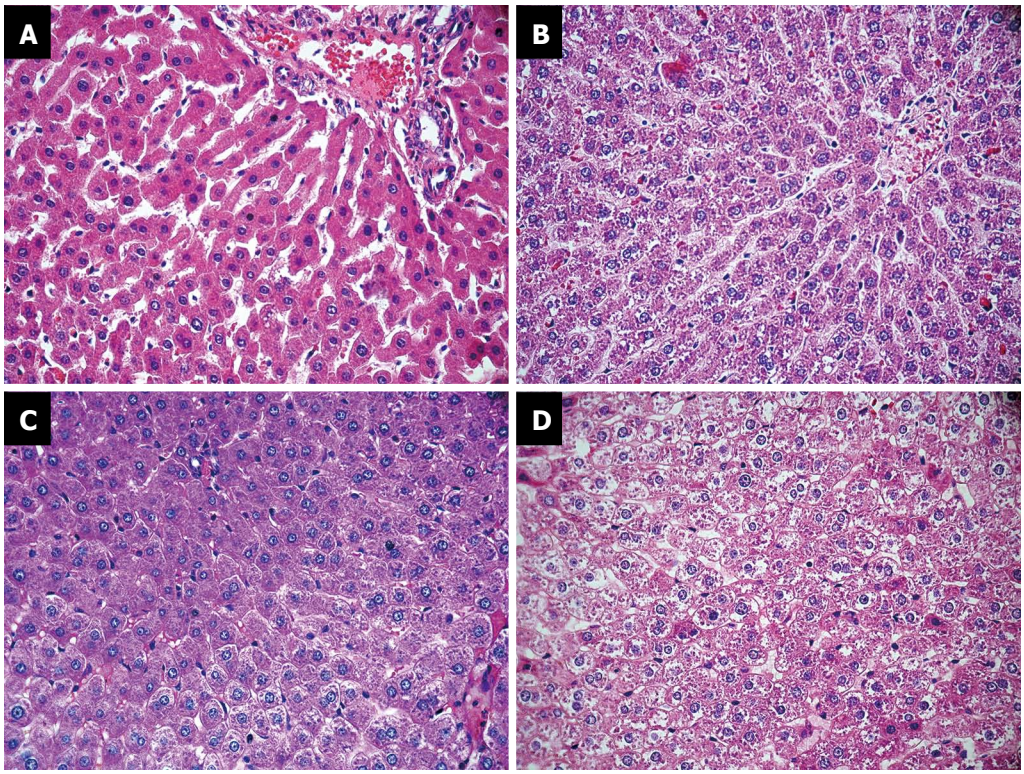

Figure 2 Staining of liver fragments at 8 wk: experimental diet group and control diet group. A: Normal central vein, functional sinusoidal dilation, and small hepatocytes in the EDG (HE, original magnification 400 ×); B: Normal central vein, sinusoidal cells, and hepatocytes in the CDG (HE, original magnification 400 ×); C: Zones without glycogen in the EDG (HE, original magnification 400 ×); D: Homogeneous distribution of glycogen in the CDG (HE, original magnification 400 ×). EDG: Experimental diet group; CDG: Control diet group; HE: Hematoxylin and eosin.

experimental diet. The stain method that we used was unable to differentiate fat from water. Other authors have recovered similar results. Lacroix *et al*[19], in an experimental study with rats fed a high-protein diet (50%) versus a normal-protein diet (14%) over the course of 6 mo, found no serious histological lesions in either group. Rats in the normal-protein diet group exhibited more microvesicular hepatic steatosis than rats in the high-protein group. Caraballo *et al*[13] showed that rats fed a high-protein and high-fat diet did not develop steatosis compared with rats fed a high-fat and low-protein diet; they concluded that the high-protein diet had an anti-steatotic effect on rat livers regardless of the amounts of others macronutrients that the rats ingested. On the other hand, Garbow *et al*[5] found hepatocellular damage, inflammatory response, severe hepatic steatosis, apoptosis, and autophagy in mice that were fed a ketogenic diet (low-carbohydrate, low-protein, high-fat) for 12 wk. Only Caraballo *et al*[13] used a stain specific to fat. For energy, high-protein diet associated gluconeogenesis improves glucose disposal from amino acids and glycogen and reduces fat deposition; neo-lipogenesis does not occur[20]. York *et al*[29] showed that a low-carbohydrate diet might be an option to treat patients with NAFLD and NASH since such a diet improves liver histology and reduces fat deposits, insulin resistance, and metabolic syndrome. Although the prevalence of NAFLD in rich countries is between 20% and 30% and this condition is considered to be part of the Metabolic Syndrome, which is responsible for high morbidity and mortality, many aspects of its pathology and treatment remain only partially understood. Apoptosis is considered an important point of NAFLD lesion and a common mechanism of hepatic injury[15,30].

We found that the amount of glycogen was likely lower in the experimental group and that both groups exhibited a heterogeneous glycogen distribution. Since the animals of each group were sacrificed in an alternating fashion, changed in glycogen content cannot be due to the duration of fasting. It was not possible to be certain that it was glycogen since no specific stain (periodic acid-Schiff reagent PAS) was conducted. Caraballo *et al*[13] reported similar results in animals fed a high-protein and high-fat diet. However, with a low-fat and low-protein diet, glycogen was not decreased and was instead concentrated in the periportal area. With a high-fat and low-protein diet, the glycogen was concentrated in the pericentral area[28]. In contrast, when Azzout-Marnich *et al*[20] compared two diets with 14% and 50% protein, respectively, glycogen levels were not different between the groups, but hey considered glyconeogenesis to be the primary pathway of the high-protein diet metabolism. Caraballo *et al* and Azzout-Marniche *et al*[20] also did not use a specific stain for glycogen.

We found no evidence for inflammation in the liver, as expected, since apoptosis (76.01%) was the primary mechanism of hepatocyte damage in the experimental group, not non-apoptotic death (23.99%). Furthermore, literature results have shown that apoptosis does not lead to a local inflammatory response[18].

Although neither group exhibited evidence of fibrosis, apoptosis may be considered not only an important mechanism of liver injury but also a contributor to liver fibrosis[27,31,32]. It is probable that if the rats had received the experimental diet for a longer period of time, fibrosis may have eventually appeared.

Additional studies are necessary to determine the exact mechanism by which changes in the percentage of macronutrients induce apoptosis. The lack of suitable nutrients may be considered to be a possible hypothesis. Gluconeogenesis, the primary metabolic pathway associated with a low-carbohydrate diet, leads to an increased production of keto acids and fatty acids, but they may not be the better energy substrate for hepatic cells[33]. When gluconeogenesis occurs, mitochondria exert important functions in energy metabolism (*i.e.*, the oxidation of fatty acids and oxidative phosphorylation for the production of adenosine triphosphate). Dysregulation of this pathway results in energy deficiencies and/or the production of reactive oxygen species that may be responsible for cell damage[34-36].

In conclusion, this study furthers our understanding of the hepatic cellular and histological changes caused by a high-protein, high-fat, and low-carbohydrate diet. The findings revealed that rats fed this diet had elevated levels of plasma transaminases and a higher percentage of nonviable cells in flow cytometry, which is evidence of hepatic lesions. Apoptosis was the principal pathway of hepatic injury. The primary findings from the optical microscopy-small hepatocytes and a decreased amount of glycogen-correlated well with changes in flow cytometry and can be attributed to modification of essential macronutrients to the liver. No inflammation or fibrosis was found in the livers of either the experimental or control animals. A better understanding of the mechanism of hepatic lesions associated with a high-protein, high-fat, and low-carbohydrate diet requires investigating the metabolic effects of diet in the liver.

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COMMENTS

Background

The authors did an experimental study comparing two groups of rats, one fed an experimental diet based on Atkins' (59.46% protein, 31.77% fat, and 8.77% carbohydrate) and the other an AIN-93M diet, so that they could study the effect of the experimental diet on hepatic metabolism. The modification of dietary components might result in hepatic cell damage since they are important targets for lesions when excessive macronutrients are absorbed and go through the intestinal mucosa to reach quickly the liver through the portal vein.

Research frontiers

The prevalence of obesity is increasing and may be considered a serious health problem. Worldwide, many people adopt various diets to lose weight without a particular orientation. The Atkins diet is a low-carbohydrate and high protein diet formulated by Dr. Robert Atkins in 1972. More recently, it was introduced for children with difficult-to-control seizures and elderly with Alzheimer's and Parkinson's diseases. Despite these indications, the effect of the diet on hepatic metabolism remains unclear.

Innovations and breakthroughs

This study demonstrated a strong association between low-carbohydrate, high-protein, and high fat diet and hepatic apoptosis, as identified by flow cytometry. These changes were correlated with alterations found by optical microscopy and may be due to metabolic changes.

Applications

A better understanding of the mechanism underlying hepatic lesions associated with this diet in rats and its effects on human metabolism is essential. The article emphasizes that the use of this diet should be used with caution for children, adults, and elders.

Terminology

Alanine aminotransferase) and aspartate aminotransferase are two enzymes found mainly in the liver that are considered hepatic markers of liver damage. Flow cytometry is a biophysical laser technology that was employed in this cell study; it detects cells in early apoptosis, late apoptosis, and nonapoptotic death.

Peer-review

Rats fed a low-carbohydrate, high-protein, and high-fat diet for 8 wk have hepatic cellular damage, as demonstrated by flow cytometry.

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ORIGINAL ARTICLE

Basic Study

Dominating expression of negative regulatory factors downmodulates major histocompatibility complex Class-Ⅱ expression on dendritic cells in chronic hepatitis C infection

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Conflict-of-interest statement: Authors declare no conflict of interest.

Data sharing statement: Technical appendix, statistical code and dataset are available from corresponding author at arora.sunil@pgimer.edu.in. Participants consent form was not taken for data sharing but the presented data are anonymized and risk of identification is low.

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Abstract

AIM: To elucidate the molecular mechanisms leading to development of functionally impaired dendritic cells (DCs) in chronic hepatitis C (CHC) patients infected with genotype 3 virus.

METHODS: This prospective study was conducted on the cohorts of CHC individuals identified as responders or non-responders to antiviral therapy. Myeloid DCs were isolated from the peripheral blood of each subject using CD1c (BDCA1)$^+$ DC isolation Kit. Monocytes from healthy donor were cultured with DC growth factors such as IL-4 and GM-CSF either in the presence or absence of hepatitis C virus (HCV) viral proteins followed by LPS stimulation. Phenotyping was done by flowcytometry and gene expression profiling was evaluated by real-time PCR.

RESULTS: Non-responders [sustained virological response (SVR)-ve] to conventional antiviral therapy had significantly higher expression of genes associated with interferon responsive element such as *IDO1* and *PD-L1* (6-fold) and negative regulators of JAK-

STAT pathway such as *SOCS* (6-fold) as compared to responders (SVR+ve) to antiviral therapy. The down-regulated genes in non-responders included factors involved in antigen processing and presentation mainly belonging to major histocompatibility complex (MHC) Class-Ⅱ family as *HLA-DP*, *HLA-DQ* (2-fold) and superoxide dismutase (2-fold). Cells grown in the presence of HCV viral proteins had genes down-regulated for factors involved in innate response, interferon signaling, DC maturation and co-stimulatory signaling to T-cells, while the genes for cytokine signaling and Toll-like receptors (4-fold) were up-regulated as compared to cells grown in absence of viral proteins.

CONCLUSION: Underexpressed MHC class-Ⅱ genes and upregulated negative regulators in non-responders indicate diminished capacity to present antigen and may constitute mechanism of functionally defective state of DCs.

Key words: Dendritic cells; Hepatitis C; Non-responders; Negative regulators; Major histocompatibility complex Class-Ⅱ genes

Core tip: The study was aimed to understand the mechanisms of dendritic cells dysfunction during chronic hepatitis C (CHC) infection. The findings highlight the association between different immune response genes and viral persistence in non-responders to antiviral therapy. Up regulation of negative regulators and down-regulation of molecules involved with antigen presentation seems to associate with non-responsiveness to antiviral therapy. Some novel pathways can be targeted to achieve better management of CHC patients.

Tomer S, Chawla YK, Duseja A, Arora SK. Dominating expression of negative regulatory factors downmodulates major histocompatibility complex Class-Ⅱ expression on dendritic cells in chronic hepatitis C infection. *World J Gastroenterol* 2016; 22(22): 5173-5182 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i22/5173.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i22.5173

INTRODUCTION

Hepatitis C virus (HCV), a positive sense single stranded RNA virus, infecting around 180 million people worldwide, is becoming a significant global health problem[1,2]. Transmitted through infected blood and body fluids, it is responsible for chronic hepatitis, which ultimately leads to life threatening liver diseases like fibrosis, cirrhosis, steatosis and finally causing hepatocellular carcinoma (HCC), thus a need for liver transplant[3]. Genotype 3 of HCV being more prevalent in South Asia, accounts for more than 50% of all the genotypes[4]. Although more patients infected with genotype 3 respond successfully to therapy as compared to genotype 1, yet approximately 25%-30% patients fail to achieve sustained virological response (SVR) and are considered as non-responders (NR) to antiviral therapy containing IFN-α and ribavirin, offered till recently[5,6].

Dendritic cells (DC) are professional Antigen-presenting cells (APC) having the unique property to induce a primary immune response[7]. Antigen uptake, processing and presentation to naïve T-cells for activation of the immune system are the main functions carried out by DC. There have been studies reported from our laboratory which showed that DC are numerically, functionally and phenotypically dysfunctional in patients infected with CHC[8,9]. The study also showed that functionally defective monocyte-derived dendritic cells (moDC) from CHC patients who did not achieve SVR, failed to reconstitute the capacity to mature, indicating that the dysfunctional status of DC in CHC patients was directly associated with the persistence of the virus.

The expression of many genes is responsible for regulation of DC maturation. They involve genes associated with antigen processing and presentation[10], interferon α, β response[11,12], cytokine signaling[13], adhesion and migration[14], phagocytosis[15], interferon responsive elements[16], anti-inflammatory process[17], negative regulators of JAK-STAT pathway[18] and genes involved in TLR mediated signaling[19].

Effective cellular immune response directed against HCV is mediated through T-cell and DC crosstalks[20]. A subdued adaptive immune response in chronic HCV patients might be due to the suboptimal antigen presentation and signaling via impaired DCs in these individuals. So, in the proposed study, we wanted to find out whether this non-responsiveness to standard anti-viral therapy in a proportion of CHC patients is associated with the virus-modulated expression of certain genes, which may culminate into dysfunctional status of DC (maturation as well as functional defects). So, we planned to investigate the expression levels of a set of selected genes in the myeloid dendritic cells (MDC) of CHC patients with the hypothesis that, an analysis of the association of immune response genes and non-responsiveness to therapy may reveal molecular mechanisms of DC dysfunction in the non-responders.

MATERIALS AND METHODS

Ethical statement

The study was approved by the Institute Ethics Committee of the PGIMER, Chandigarh (Reg. No. NKG/947). An informed written consent was obtained from all the subjects before taking blood samples.

Subjects and sampling

A total of 20 CHC patients were recruited for the study. Patients were divided into two groups on the basis of response to therapy in terms of SVR *i.e.,* HCV RNA negative at 24 wk after cessation of antiviral therapy. Patients achieving SVR (SVR+ve) were considered as "Responders" whereas those who failed to achieve SVR (SVR-ve) were termed as "non-responders". Responders (n = 10, MDC-R) and Non-responders (n = 10, MDC-NR) were recruited on the basis of inclusion and exclusion criteria. Inclusion criteria included patients positive for anti-HCV antibodies and serum HCV RNA, HCV RNA genotype 3 only, no prior history of any treatment for HCV, negative for auto-antibodies (ANA, SMA, LKM, AMA and PCA) and non-viral factors (alcoholism, inherited metabolic disorders). Exclusion criteria included patients with HBV, HCV genotype 1, 2 or 4, HIV and other co-infections, patients with regular use of hepato-toxic drugs and alcohol intake and any evidence of auto-immune or metabolic disease. Venous blood was taken in heparin vacutainer vials (BD) from each recruited patient in the hepatology clinic of PGIMER, Chandigarh. Age and sex matched healthy volunteers were recruited as control subjects (HC; n = 10). Inclusion criteria for HC included those subjects who had normal liver function tests with no history of jaundice or viral hepatitis infection in the past.

PBMC isolation and enrichment of myeloid dendritic cells using magnetic beads

Plasma was stored at -80 ℃ before isolation. From heparinised blood, peripheral blood mononuclear cells (*PBMCs*) were isolated by ficoll-hypaque density gradient centrifugation using Hisep (Himedia, Mumbai, India). MDC enrichment was performed by using CD1c (BDCA-1)$^+$ Dendritic Cell Isolation Kit (MiltineyiBiotec, Germany) following manufacturer's instructions. Briefly, the procedure included two steps: In the 1st step, CD1c (BDCA-1) expressing B cells labeled with CD19 magnetic microbeads got depleted by separation over a MACS column placed in a magnetic field of a MACS Separator. In the second step, CD1c (BDCA-1)$^+$ MDC labeled with CD1c-Biotin and Anti-biotin microbeads in B cell depleted flow-through fraction were retained within the column and eluted after removing the column from magnetic field. These cells (MDC) were used for further experiments.

Flow cytometric analysis for purity check

PBMCs and MDCs (10 μL each) were stained with fluorochrome-labeled antibodies (2 μL): Allophy-cocyanin (APC)-conjugated anti-HLA-DR, Fluorescein isothiocyanate (FITC)-conjugated Lineage Cocktail 1 (Lin1: CD3, CD14, CD16, CD19, CD20, CD56) and Phycoerythrin-Cy5 (PE-Cy5) - conjugated anti-CD11c from BD Biosciences (San Jose, CA, United States) for 15 min in the dark. Cells washed with staining buffer for 5 min at 1400 rpm were re-suspended in buffer for acquisition on Flowcytometer (FACS Calibur, BD, United States). Percent purity was calculated.

Generation of monocyte-derived DCs from HCs PBMCs

Monocyte-derived dendritic cells (moDCs) were derived according to the method described by Romani *et al*[21] and modified in our laboratory[8]. Cells were cultured in the presence (moDC-Ag) or absence (moDC-N) of HCV viral proteins. Briefly the PBMCs were isolated from venous blood as described above. Cells were suspended in RPMI 1640 medium (Sigma-Aldrich) and monocytes were made to adhere for 2 h at 37 ℃ (Plate adherence method). After incubation, non-adherent cells were removed. Adherent cells were cultured in the DC culture medium (DCCM) consisting of RPMI 1640 supplemented with: 2 mmol/L L-glutamine, 5 mmol/L HEPES buffer, 100 IU/mL penicillin and 100 μg/mL streptomycin, 10% fetal bovine serum (GIBCO), 20 ng/mL recombinant human GM-CSF (Peprotech Asia) and 20 ng/mL recombinant human IL-4 (Peprotech Asia) at 37 ℃ in a humidified incubator with CO_2 volume fraction, 50 mL/L CO_2 for six days. The cells were cultured in different sets as: either in presence or absence of viral proteins: core, NS3, NS4 and NS5 (Peprotech Asia). At the end of six days, these moDCs were stimulated with bacterial lipopolysaccharide (LPS) and further cultured for 48 h in maturation cocktail which comprised of DCCM with LPS (500 ng/mL). On the 8th day, moDCs were harvested and gene expression studies were carried out.

Gene expression analysis (RNA extraction, cDNA preparation and real time PCR)

MDCs and moDCs were centrifuged and dissolved in 1 mL TRIzol (Sigma, United States). RNA was extracted and reverse transcribed to cDNA using the RT^2 First Strand Kit (Qiagen, Germany) according the manufacturer's protocol, and cDNA was stored at -20 ℃ till further use. A custom PCR array (RT^2 Custom Profile PCR Array Human, Qiagen) was designed which included a panel of immune-stimulatory genes (ISGs) and genes involved in DC functioning (Table 1). Real-time PCR was undertaken using RT SYBR Green Master Mix (Qiagen, Germany) in a 96-well PCR plate pre-dispensed with primers in a Light Cycler 480 (Roche, Germany). Values were normalized against housekeeping genes (*GAPDH, β-actin*) in the same sample. Each experiment included positive PCR control (PPC), reverse transcription control (RTC) and human genomic DNA contamination (HGDC) control. Ct values were obtained for calculation of delta-CtCt and further analysis.

Statistical analysis

Statistical analysis for viral load (baseline and 4 wk) and other clinical features were done using GraphPad Prism software v 5.03 statistical package. Parametric

Table 1 List of selected genes for custom PCR array

CD209	CSF1R	ADAMDEC1	PDCD1	HLA-DPB1	HLA-DQA1	HLA-DQB1	HMOX1	ITGB2	CD40	*CD80*	*CD86*
CD83	*LY75*	*LAMP3*	*ARHGDIB*	*CCL5*	*CCL8*	*TLR2*	*CCL22*	*CCR7*	*CXCR3*	*CXCR4*	*CXCL6*
CXCL9	*CXCL10*	*CXCL11*	*CXCL12*	*CXCL16*	*ITGAX*	*ICAM1*	*VCL*	*TLR8*	*NFKB1*	*NFKB2*	*CD1A*
CD1B	*CD1C*	*CD52*	*S100A4*	*RELB*	*IDO1*	*CD274*	*IFNAR1*	*IFNAR2*	*IRF1*	*IRF3*	*CD44*
IRF7	*IRF9*	*STAT1*	*STAT2*	*ADAR*	*EIF2AK2*	*IFI6*	*IFI27*	*IFI35*	*OAS1*	*OAS2*	*OAS3*
PRKRA	*SOD2*	*MX1*	*MX2*	*ISG15*	*ISG20*	*IFIT1*	*FAS*	*LITAF*	*IFIT3*	*IFITM1*	*ITIH2*
GBP1	*GBP2*	*PIAS1*	*PIAS2*	*SOCS1*	*SOCS2*	*SOCS3*	*SOCS4*	SOCS5	IL28B	TRIM22	RARRES3
TAP1	*TAP2*	*RELA*	*TLR3*	*TLR4*	*TLR7*	*TLR9*	*GAPDH*	*ACTB*	*HGDC*	*RTC*	*PPC*

Table 2 Clinical characteristics of study subjects

Parameter	Responder	Non-responder
Mean viral load (IU/mL)	6.09 ± 0.29	5.97 ± 0.81
Mean age (yr)	42.0 ± 2.8	47 ± 2.9
Male/Female	6/4	8/2
Mean TB/CB (mg/dL)	0.75 ± 0.10	1.25 ± 0.20
Mean AST (U/L)	94.77 ± 23.40	102.3 ± 12.7
Mean ALT (U/L)	155.4 ± 45.2	111.2 ± 18.3
Mean AP (U/L)	94.50 ± 8.20	160.00 ± 24.44
Mean A/G (mg/dL)	1.25 ± 0.10	1.74 ± 0.10
(Fibrosis) Median LSM (kPa)	6.10	23.50 (P = 0. 01)

TB/CB: Total bilirubin/conjugated bilirubin; AST: Aspartate transaminase; ALT: Alanine transaminase; AP: Alkaline phosphatases; A/G: Albumin/globulin; LSM: Liver stiffness measurement.

and non-parametric *t*-tests were carried out and $P < 0.05$ was considered significant. For flow cytometry results, Cellquest software (BD Biosciences, United States) was used. Analysis of up-regulated and down-regulated genes was done using web based online software RT^2 Profiler PCR array data analysis version 3.5 software. To check interactions and associations between different genes, string software available online was used.

RESULTS

Clinical and demographic details of patients

A total of 20 patients were recruited for the study. Their clinical and demographic parameters like gender, age, genotype, liver enzyme (AST-ALT) levels, total bilirubin/conjugated bilirubin, Alkaline Phosphatase (ALP) levels were recorded (Table 2). At the baseline, there was no significant difference in the viral loads of responders vs non-responders, but when compared between baseline *vs* 4 wk (at RVR - rapid virological response) the viral load became undetectable in responders, while remained detectable in non-responders although was significantly decreased (Figure 1). Also, the degree of liver fibrosis (LSM - liver stiffness measurements) which provides useful information in prognostication, therapeutic planning, and assessment of the impact of treatment in chronic liver diseases, was significantly increased in non-responders ($P < 0.05$), which suggests that the persistence of virus in the liver leads to cirrhosis of the liver.

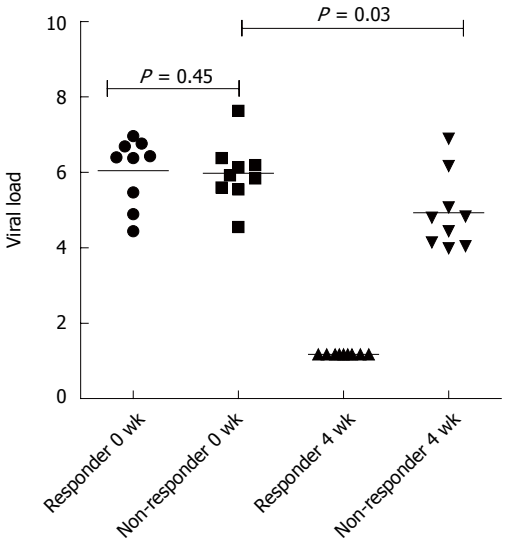

Figure 1 Difference between viral load in responders to antiviral therapy (at 0 and 4 wk) *vs* non-responders (0 and 4 wk). No significant difference in the viral loads of responders vs non-responders at baseline, but when compared between baseline *vs* 4 wk (at RVR - rapid virological response) the viral load became undetectable in responders, while remained detectable in non-responders.

Flow cytometric analysis of MDC

For phenotyping and purity of the isolated MDC, the cells negative for Lineage (CD3, CD14, CD16, CD19, CD20, CD56) and dual positive for CD11c and HLA-DR were gated. Percent enrichment of MDC was 65% after magnetic sorting as compared to 10% in PBMCs before sorting.

Gene expression profiles by PCR array

The gene expression profiles of the selected genes (as in Table 1) using custom-designed PCR array are shown in the heat map of genes indicating the differentially expressed genes (Figure 2). The genes upregulated or down-regulated are shown in Figure 3.

Upregulated genes

Non-responders (MDC-NR) *vs* Responders (MDC-R) group: Genes involved in negative signaling of JAK-STAT pathway, such as suppressor of cytokine signaling (*SOCS1*; six-fold, *SOCS2, SOCS4* and *SOCS5* all two-fold) and genes involved with down-modulation of immune response such as Indoleamine 2,3-Dioxygenase (*IDO1*) and Programmed death-

Figure 2 Heat map showing the expression of all the genes in different groups recruited. Graphical representation showed up-regulated and down-regulated genes in all the recruited groups. NR: Non-responder; HC: Healthy controls; HT: Healthy treated; RESP: Responder.

ligand 1 (*PD-L1*) were found to be significantly upregulated (two-fold or more) in non-responders as compared to responders to therapy (Figure 4).

Further, the genes for antiviral innate response such as TLR, ISGs and JAK/STAT pathway were also found to be up-regulated in non-responders as compared to responders to antiviral therapy.*TLR3* which is activated by viral RNA (HCV RNA) was four-fold up-regulated whereas *TLR4* and *TLR7* showed two-fold up-regulation in non-responders. The genes for Interferon regulatory factors (*IRF 7* and *IRF 9*) and the Interferon stimulatory genes (*ISG15* and *ISG20*) were also found to be significantly (six-fold) upregulated in non-responders. Further, the genes involved in JAK-STAT signaling, the *STAT1* and *STAT2,* showed two-fold upregulation along with the increased expression of IFN-induced proteins with tetratricopeptide repeats (*IFIT1*; six-fold, *IFIT3*; two-fold), IFN-Inducible transmembrane family (*IFITM1*; two-fold), Interferon-induced GTP-binding protein encoding gene (*MX1*, *MX2*; both two-fold), 2′,5′-oligoadenylate synthetase (*OAS1*; six-fold, *OAS2*; two-fold), IFN-inducible genes (*IFI6*; two-fold, *IFI27*; two-fold, *IFI35*; four-fold), Adenosine deaminase acting on RNA (*ADAR*; two-fold) and eukaryotic translation initiation factor 2-alpha kinase 2 (*EIF2AK2*; two-fold). Also gene associated with apoptosis such as Fas cell surface death receptor (*FAS*) showed increased expression (two-fold) in non-responders to antiviral therapy.

moDC from healthy donor differentiated in presence (moDC-Ag) or absence (moDC-N) of viral proteins: Sixteen genes were upregulated in the moDC differentiated from monocytes grown in presence of HCV viral proteins as compared to the cells grown in absence of proteins. Amongst these, included the chemokine and their receptor genes (*CXCR3*, *CXCR6*, *CXCL12*, *CCL8*; all two-fold) and Toll-like receptor genes (*TLR2*, *TLR4*, *TLR9*; all two-fold).

Downregulated genes

Non-responders (MDC-NR) *vs* Responders (MDC-R) group: The genes downregulated in non-responders as compared to responders included the genes belonging to MHC-Class Ⅱ family (*HLA-DPB1*, *HLA-DQA1*, *HLA-DQB1*) and Superoxide dismutase (*SOD*), the enzyme involved in transforming toxic superoxide anion radicals into hydrogen peroxide and oxygen for protecting DNA from oxidative stress showed two-fold reduced expression in non-responders as compared to responders (Figure 4).

DCs from healthy donor grown with (moDC-Ag) or without (moDC-N) viral proteins: A decreased expression of 21 genes in the cells grown in presence of viral proteins was observed as compared to the cells grown in absence of proteins. The genes found down-regulated (two-fold) include the ones involved in innate response and Interferon signaling (*EIF2AK2, IFI27, OAS1, OAS2, MX1, IFIT1, IFIT3, GBP1, GBP2, ISG20*); the genes involved with DC maturation (*CD83, LY75, LAMP3*) and genes involved in delivering co-stimulatory signals to T-cells (*CD40, CD80, CD86*) (Figure 4).

DISCUSSION

Non-responsiveness to antiviral therapy has been linked to defective phenotype of MDC by previous reports including our laboratory[8,9]. This indicates a direct association of immune defects with response to treatment in CHC, which could be attributed to many reasons such as (1) defect in IFN-α interactions with its receptors on MDCs; (2) defect in signal transduction machinery after this interaction; and (3) abnormal expression of certain transcription factors and immune response genes which are involved with the activation and maturation of DCs.

Although it has already been reported that there are functional and maturation defects in MDCs during CHC infection, yet the molecular mechanisms involved have not been fully elucidated[8]. The present study was designed with a view to understand these mechanisms and the role of different immune response genes that are involved in regulation of DC functions and may be associated with non-responsiveness to therapy and viral persistence during CHC. In order to achieve the objectives, gene expression profiles were studied in MDC isolated from the peripheral blood of CHC patients put on standard anti-viral treatment consisting of

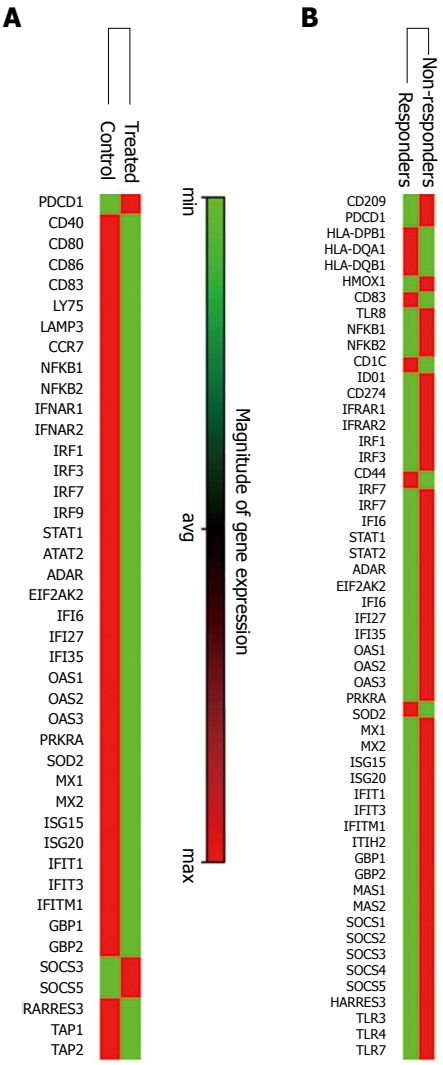

Figure 3 Clustergram showing up-regulated and down-regulated gene expression in dendritic cells of A: moDCs from healthy volunteer differentiated in presence of HCV viral proteins (Test group) and moDCs differentiated in absence of viral antigens (control group); B: Responders and non-responders. Graph showing the differentially expressed genes in different groups. Green represents the lower expression of a particular gene and red represents the higher expression of a particular gene in that particular group as compared to control.

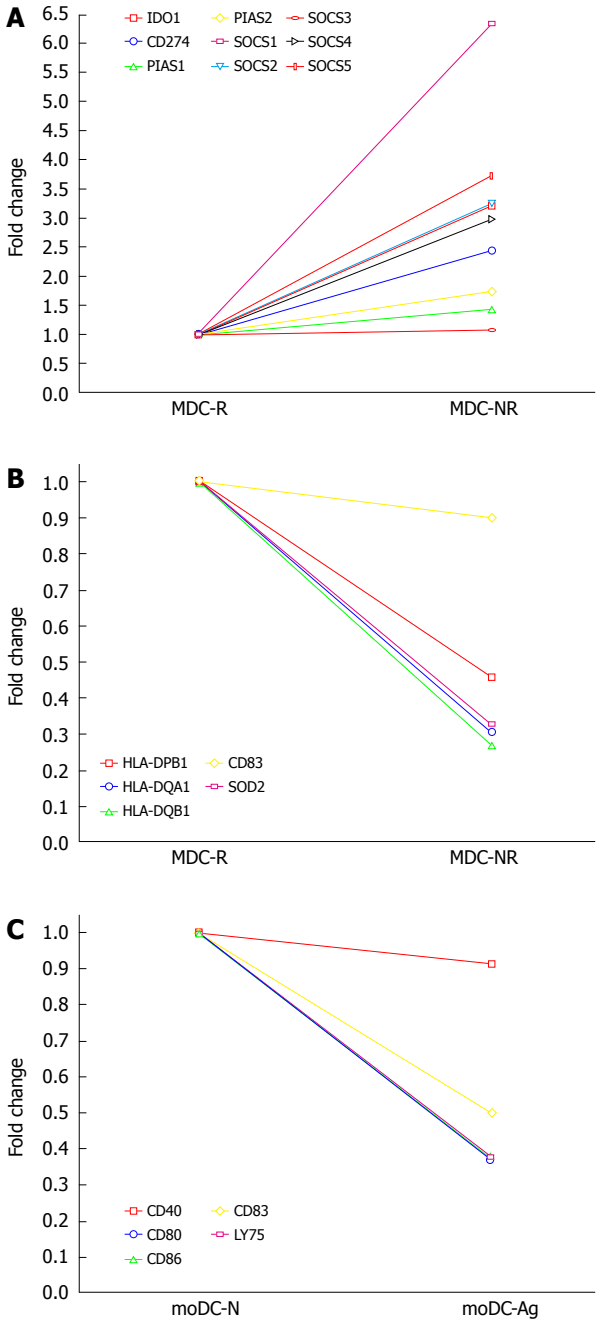

Figure 4 Multigroup plot showing A: up-regulation of down modulatory genes (negative regulators) in MDC-NR (non-responders) as compared to MDC-R (responders); B: Down-regulated genes in MDC-NR (Non-responders) as compared to the MDC-R (Responders); C: Down-regulation of genes involved with DC maturation and co-stimulatory molecules in the cells differentiated in the presence of viral antigens (moDC-Ag) as compared to cells without antigens (moDC-N). The genes which are negative regulators of JAK-STAT such as suppressors of cytokine signaling (SOCS), protein inhibitors of activated STATs are up-regulated in non-responders as compared to responders whereas the genes which belong to MHC class Ⅱ family such as HLA-DPB1, HLA-DQA1 and HLA-DQB1 and dendritic cell maturation marker such as CD83 is down-regulated in non-responders as compared to responders.

Type 1 IFN and ribavirin, some of those who achieved SVR were termed "responders" and those who did not achieve SVR were termed "non-responders". The differentially expressed genes were identified after analysis of the expression profile results. Further, these findings were confirmed in an *ex vivo* moDC model where gene expression profiles were analyzed in monocytes from a healthy donor, differentiated to DC, either in presence or absence of some HCV proteins, using same custom-designed PCR array. Interestingly results from both these experiments although not exactly overlapping, yet revealed the set of genes down-regulated in "non-responders" or in cells grown in presence of viral proteins were those, which are involved with DC maturation and function. Similarly the genes that were found to be up-regulated in these cells were mainly of the negative regulators of DC functions suggesting that the continuous presence of virus or viral proteins in individuals infected with HCV, would facilitate the development of functionally

defective phenotype of DCs in these individuals.

The monocytes cultured and differentiated in the presence of HCV viral proteins to dendritic cells in a culture system *ex vivo,* in the present study, induced the development of a defective phenotype of DCs with hampered maturation capabilities in a similar manner and also confirmed the findings from CHC patient experiments as described above. The analysis of gene expression profiles of these cells revealed downregulated expression of some important genes associated with DC maturation (*LAMP3* and *LY75*), co-stimulatory signaling (*CD80* and CD86) and many immune-stimulatory genes, the ISGs (*EIF2AK2*, *IFI27*, *OAS1*, *OAS2*, *MX1*, *ISG20*, *IFIT3*, *IFIT1*, *GBP1* and *GBP2*), which are the first line of defense in innate antiviral immunity, suggesting that persistence of HCV down-modulates the host defense mechanisms and make conditions favorable for its own survival. These results are consistent with the earlier reports, which also indicated that different HCV viral proteins disrupt the host IFN signaling and ISGs to establish chronic infection[22].

Entry of the virus in the host results in up-regulation of many TLRs like *TLR2*, *TLR3*, *TLR4*, *TLR7*, *TLR8* and *TLR9* on PBMCs and monocytes[23]. The expression of *TLR3*, *TLR4* and *TLR7* was also found to be increased on the MDC of non-responders in our study, suggesting the immune activation due to the constant presence of viral RNA. Besides, *RARRES* (Retionic acid receptor responder protein 1), which is also activated by viral RNA, was also upregulated in non-responders. The *TLR7* and *RARRES* cause *IRF7* activation and induction of Type1 IFN gene, leading to activation of JAK-STAT signaling and upregulated expression of *STAT1*, *STAT2* and *IRF9*, which further lead to enhanced expression of ISGs namely *IFIT1*, *IFIT3*, *ISG15*, *ISG20*, *ADAR*, *GBP1*, *PRKRA*, *EIF2AK2* (PKR), *IFITM1*, *MX1*, *MX2*, *OAS1*, *OAS2*, *IFI16*, *IFI27* and *IFI35*. The administration of exogenous IFN and upregulation of ISGs may be effective to a certain limit because virus replication and copy number gets significantly reduced from baseline to week 4 (RVR) in these patients, but complete removal was not achieved as viral load was still detectable, which suggests that there are other factors that are associated with the persistence of the virus.

The possible reasons may be attributed to dampening of the immune response (functionally impaired immature CD4+ cells) by Type 1 IFN, which leads to impaired T-cell immunity as evident in these patients. Moreover the genes important for optimal antigen presentation like MHC-Ⅱ (*HLA-DP*, *HLA-DQ*) and co-stimulatory molecules (*CD80*, *CD86*) as well as the homing receptors like *CCR7* were all found to be down-regulated in MDC from non-responder patients as compared to responders in our study, which again supports the hypothesis that such maturation defective DCs with hampered antigen-presenting and migration capabilities would be responsible for the generation of functionally impaired set of immature T-cells, which are incapable of clearing the virus in CHC. Also, it has been reported earlier that excessive or prolonged IFN-αβ signaling is associated with severe disease in HIV infection and favors the replication of virus in the host[23]. Although HCV has different mechanisms to down-regulate Type 1 IFN production, still ISGs are induced in infected hepatocytes in most of the chronically infected CHC patients. Earlier reports suggest that HCV patients having high pre-existing levels of ISGs are less likely to respond to IFN-α therapy in comparison to those with lower levels[24].

Besides, the genes for factors such as *SOCS1*, *SOCS2*, *SOCS4* and *SOCS5* which negatively regulate the inflammatory pathways such as JAK-STAT signaling[25]; *PDL1* responsible for exhaustion of T-cell function and its blocking on DCs shown to enhance T-cell activation[26,27]; *IDO1* which alters DC by decreasing its APC function and capable of suppressing local T-cell immune responses and promoting systemic tolerance[28], were all up-regulated in non-responders. Our findings corroborate the study reported earlier that up-regulation of PD-1 and SOCS-1 inhibitory molecules mediates functional impairment of the early immune response during HCV infection[29].

The expression of CD209 (DC-SIGN), a molecule expressed more on immature DCs and involved in innate immune responses also plays a critical role in viral pathogenesis, was also upregulated in non-responders[30,31]. Immature DCs bind more strongly to E1 and E2 (HCV Envelope proteins) through DC-SIGN with a difference in internalization pathway. These HCV viral like particles are targeted to non-lysosomal compartment in immature DCs and are protected from lysosomal degradation[32]. Thus, HCV may use DC-SIGN as an entry portal and facilitate viral infection of nearby hepatocytes and also use these DCs as reservoirs resulting in establishment of viral infection. HIV gp120 also binds to DC-SIGN and results in horizontal and vertical transfer and also helps in spreading the virus in the host[33].

HCV induces chronic increase in hepatic oxidative stress which plays an important role in pathogenesis of HCV[34]. Expression of genes of the factors involved with stress conditions such as Heme Oxygenase (*HMOX1*) was up-regulated in non-responders, suggesting higher oxidative stress in these patients. Since the *HMOX1* catabolizes heme to bilirubin, this might be responsible for significantly higher bilirubin levels observed in non-responders as compared to responders. The *HMOX1* being mainly expressed in immature DCs and its overexpression induces down-regulation of co-stimulatory molecules on DCs, might be responsible for inhibition of T-cell proliferation in CHC[35]. On the other hand, *SOD* responsible for transforming toxic superoxide anion radicals, showed down-regulated expression in non-responders resulting in increased levels of reactive oxygen species (ROS) and oxidative stress in these patients, might

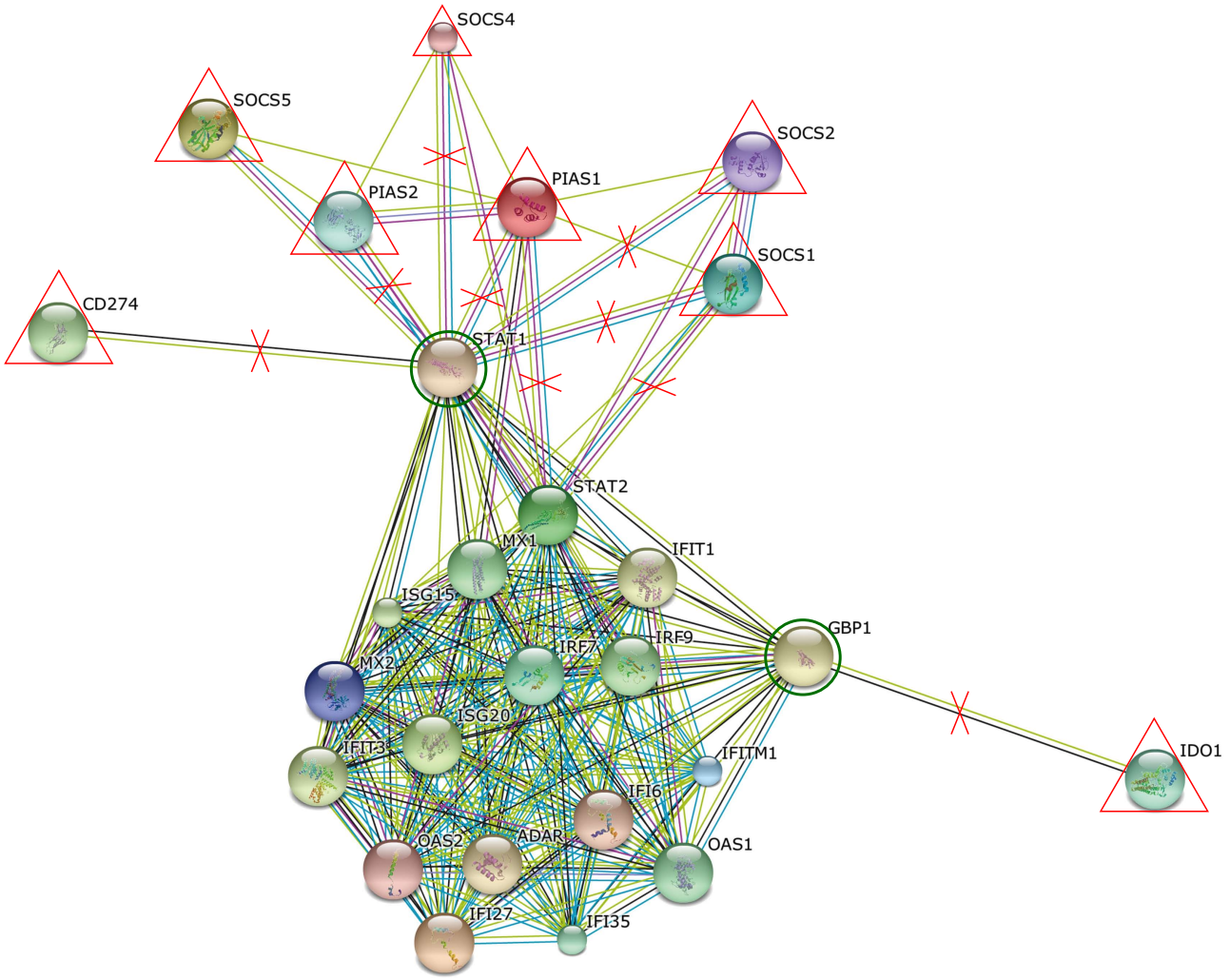

Figure 5 Interactions between different interferon stimulatory genes and negative regulatory genes of immune system. Using string software, possible interaction pathways between different Interferon Stimulatory genes and Negative regulatory genes were drawn. Negative regulators such as SOCS, PIAS when upregulated results in the inhibition of *STAT* and *GBP* genes which in turn result in the induction of transcription of many genes involved in innate immunity and interferon signaling such as *IRF7*, *IRF9* and *ISG20*. ISG: Interferon stimulatory genes.

be responsible for increased apoptosis of cells, which is also supported by up-regulated expression of *FAS* gene observed in these patients in our study. Thus, these patients are unable to destroy superoxide anion radicals, which are normally produced within the cells and are toxic to biological systems. Earlier report has also shown increased levels of *HMOX1* and decreased levels of *SOD2* in PBMC of patients with CHC[36].

In summary, our study indicates that there is up-regulation of negative regulators and down-regulation of molecules involved with maturation and antigen-presentation on DCs of non-responders. This imbalanced state, possibly modulated by the continuous replication of HCV, results in the generation of maturation-defective phenotype of DCs which are not capable of presenting the viral antigens to the naïve T-cells and lead to the generation of functionally defective immature T-cells incapable of clearing the virus (Figure 5). Whether this defective state of DCs in these patients is the cause or effect of viral persistence, is not really clear, but possibly this vicious cycle might be the cause of non-responsiveness to anti-viral therapy. Never the less, the study points to some novel pathways that may be targeted to achieve better management of this chronic disease.

COMMENTS

Background

In patients infected with hepatitis C, it had already been reported that dendritic cells are numerically, functionally and phenotypically dysfunctional. Also functionally defective monocyte-derived dendritic cells (DCs) from chronic hepatitis C (CHC) patients who did not achieve sustained virological response (SVR) failed to reconstitute the capacity to mature, indicating the dysfunctional status of DC in CHC patients, however the molecular mechanisms regulating this defect have not been elucidated.

Research frontiers

Previous experiments have indicated that CHC patients having dysfunctional dendritic cells led to therapy non-responsiveness in these patients.

Innovations and breakthroughs

The expression profile of selected genes related to hepatitis C virus (HCV) infection have been studied in various hepatocytes cell lines but, the role

of dendritic cells in non-responsiveness to antiviral therapy has not been elucidated as yet.

Applications

The molecular profile of dendritic cells on their role to standard therapy may be used in better prognosis and will help in designing newer therapeutic modalities that might help in better management of non-responders who do not respond to extended regimens to antiviral therapy.

Terminology

SVR - HCV RNA negative 24 wk after cessation of treatment. It is the best predictor of a long-term response to treatment.

Peer-review

The paper of Tomer S *et al* discusses the effects of HCV on DCs isolated from PBMC of IFNα-treated HCV patients. The comparisons in gene expression have been done between the responders and non-responders to treatment *vs* DC from healthy donors exposed or not to HCV proteins. This is an interesting attempt to elucidate the role of HCV-infection in impairment of DC function and to link this situation to non-responsiveness to IFNα treatment, which provides a promising connection to translational research.

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ORIGINAL ARTICLE

Basic Study

miR-106b promotes cancer progression in hepatitis B virus-associated hepatocellular carcinoma

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Abstract

AIM: To investigate the effect of miR-106b on tumor progression in hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC).

METHODS: A total of 120 patients who underwent liver resection for HCC at National Cheng Kung University Hospital were enrolled in the present study. MicroRNA (miRNA) array was first used to screen the miRNA expression profiles in HCC patients. The clinical records were retrospectively analyzed, and correlations with the miRNA expression profiles were evaluated. The mRNA expression levels of the miR-106b-25 cluster (miR-106b, miR-93 and miR-25), and MCM7 in tumor and non-tumor samples were quantitated using quantitative real-time reverse transcription-polymerase chain reaction (q-RT-PCR) analysis, and correlations in the levels of miR-106b, miR-93 and miR-25 expression were calculated. Kaplan-Meier overall and disease-free survival rates of HBV-associated HCC patients were analyzed using the log-rank test based on miR-106b expression. The comparison of the miR-106b expression levels in patients with different clinical outcomes was analyzed using Mann-Whitney *U* tests. Furthermore, a hepatitis B virus X protein (HBx) expression plasmid was transfected into Huh7 and Hep

3B cells. The expression levels of the miR-106b-25 cluster and MCM7 in HBx-expressing Huh7 and Hep 3B cells were detected using q-RT-PCR.

RESULTS: miRNA array screening showed that miR-106b and its cluster, miR-93 and miR-25 were up-regulated in HCC patients ($P < 0.01$). The value of miR-106b expression in HBV-associated HCC patients was significantly higher than that in HCV- ($P < 0.05$) or non-B/non-C- ($P < 0.001$) associated HCC patients. The expression of the miR-106b-25 cluster was significantly higher in tumor tissue ($P < 0.001$) and associated with the host gene, MCM7, in clinical specimens from HBV-associated HCC patients. Furthermore, the expression levels of miR-106b, miR-93 and miR-25 were positively correlated in HBV-associated HCC tissues (miR-106 *vs* miR-93, $r = 0.75$; miR-93 *vs* miR-25, $r = 0.69$; miR-106b *vs* miR-25, $r = 0.33$). The overall and disease-free survival curves showed that high-miR-106b expression was correlated with the poor prognosis of HBV-associated HCC. HCC differentiation was significantly correlated with miR-106b expression ($P < 0.05$). Lower miR-106b expression levels resulted in the well differentiation of HCC. Moreover, the expression of the miR106b-25 cluster and MCM7 was up-regulated in Huh7 and Hep 3B cells after transfection with the HBx expression plasmid.

CONCLUSION: The data obtained in the present study suggests that HBx enhances miR-106b transcription to promote tumor progression in HBV-associated HCC.

Key words: miR-106b; Hepatitis B virus; Hepatocellular carcinoma; Tumor progression; Hepatitis B virus X protein

Core tip: The role of miR-106b in tumor progression of hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC) and how it be regulated are still unclear. In this study, we analyzed the expression levels of miR-106b in HBV-associated HCC tissues and correlated the data with clinical records of patients. Our results indicated that miR-106b expression was up-regulated and related with tumor progression in HBV-associated HCC. In addition, hepatitis B virus X protein may contribute to enhance the transcription of miR-106b. These findings provide potential diagnostic and therapeutic targets for HBV-associated HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) ranks among the 10 most common cancers in the world and is a major cause of cancer death in Southeast Asian countries[1,2]. The carcinogenesis of HCC is a multi-factor, multi-step and complex process, associated with chronic and persistent hepatitis B virus (HBV) infection[3,4]. Chronic hepatitis infection causes liver inflammation damage, subsequent fibrosis, liver cell regeneration and liver cell proliferation leading to the malignant transformation of the liver[5]. Most HCC patients die as a result of the rapid tumor progression and hepatic resection or transplantation is the only potential curative treatment for HCC patients when the HCC is diagnosed early[6]. However, the effective diagnostic and therapeutic targets remain unclear.

Several mechanisms of HBV-related tumorigenesis have been proposed[5]. HBV X (HBx) protein has recently been implicated as an oncoprotein in HBV-related tumorigenesis and HCC progression[7,8]. Previous studies have shown that HBx modulates cytoplasmic signal transduction pathways, such as Ras/Raf-1, through the transactivation of cellular signaling molecules to promote HCC proliferation[9].

MicroRNAs (miRNAs) are small non-protein coding gene (19-22 or 19-25 nucleotides) with important role in the regulation of gene expression at the post-transcriptional level[10]. Studies have demonstrated that miRNA plays a role in the regulation of fundamental cellular processes, including development and proliferation, cell fate determination and apoptosis[10,11]. Nearly 60% of human genes are controlled through miRNAs[11]. Several studies have shown that miRNA might affect on numerous types of cancer, and the dysregulation of miRNA has been associated with certain cancer types[11-14]. The dysregulated miRNA promotes or suppresses tumorigenesis through the down-regulation tumor suppressor gene or oncogene expression[15,16]. The miR-106b-25 polycistron is located within intron 13 of the minichromosome maintenance protein 7 (MCM7) genes on chromosome 7q22.1[17]. The results of previous sequence study indicated that miR-106b-25 is homologous with the known oncogene miR-17-92[18]. Previous studies have shown that the miR-106-25 cluster is overexpressed as a group of oncogenic miRNAs in many cancer types including prostate cancer, breast cancer, and gastric cancer[14,19-21]. MCM7, the host gene of the miR-106b-25 cluster, belongs to a family of the minichromosome maintenance (MCM) complex, comprising six replication proteins including MCM2, MCM3, MCM4, MCM5, MCM6 and MCM7 (termed MCM2-7). Previous studies have implicated MCM7 in the replication licensing and synthesis of DNA[22,23]. The expression of MCM7 can be a prognostic indicator in diverse cancers, such as prostate cancer, ovarian cancer, endometrial cancer,

Table 1 Characteristics of hepatocellular carcinoma patients in the present study[1]

Characteristics	Patient numbers *n* (%)		
	1st Cohort (*n* = 12)	2nd Cohort (*n* = 108)	Total (*n* = 120)
Gender			
Male	8 (67)	86 (80)	94 (78)
Female	4 (33)	22 (20)	26 (22)
Age (yr)			
< 50	0 (0)	15 (14)	15 (13)
⩾ 50	12 (100)	93 (86)	105 (87)
Viral infection			
HBV	5 (42)	108 (100)	113 (94)
HCV	6 (50)	0 (0)	6 (5)
Non-B/Non-C	1 (8)	0 (0)	1 (1)
HCC differentiation			
Well	1 (8)	20 (19)	21 (18)
Moderate	8 (67)	76 (70)	84 (70)
Poor	3 (25)	11 (10)	14 (11)
Unknown		1 (1)	1 (1)
Pathological staging			
Stage Ⅰ	1 (8)	39 (36)	40 (33)
Stage Ⅱ	9 (75)	51 (47)	60 (50)
Stage Ⅲ	2 (17)	18 (17)	20 (17)

[1]A total of 120 hepatocellular carcinoma (HCC) patients were divided into two cohorts. A total of 12 HCC patients with distinct types of HCC were included in the 1st cohort, and the remaining 108 hepatitis B virus (HBV)-associated HCC patients were enrolled in the 2nd cohort.

etc[24-26]. Moreover, the dysregulation of MCM7 might be involved in tumor development and associated with the miR-106b-25 cluster.

In the present study, we analyzed the expression levels of miR-106b in HBV-associated HCC tissues and correlated the data with the clinical records of patients to clarify the role of miR-106b in tumor progression and regulation in HBV-associated HCC. These results indicated that miR-106b expression is up-regulated and associated with tumor progression in HBV-associated HCC. In addition, HBx might enhance miR-106b transcription. Thus, these findings highlight a potential diagnostic marker and a therapeutic target for HBV-associated HCC.

MATERIALS AND METHODS

Patients and HCC tissue

A total of 120 patients who underwent liver resection for HCC at the National Cheng Kung University Hospital from September 2012 to July 2015 were enrolled in the present study. Informed consent regarding use of specimens for this research was obtained from all patients and all protocols were reviewed and approved through the National Cheng Kung University Hospital Institutional Review Board. The patients were regularly followed up at clinical visits every 1 to 3 mo after curative surgery. The patients included 94 (78%) males and 26 (22%) females ranging in the age from 34 to 90 years (mean age 61.6 years). The median follow-up time was 35 mo (range, 1 to 118.8 mo). At the end of the follow-up, 25 patients had died of disease. HCC patients were divided into two study cohorts: 12 patients with distinct types of HCC were included in the 1st cohort to screen the miRNA expression profile using miRNA array, and the other 108 HBV-associated HCC patients were enrolled in the 2nd cohort for further analysis of the role of miR-106b in HBV-associated HCC. The characteristics of the HCC patients are listed in Table 1. The HCC tissue specimens were collected during surgery. The clinical records of the patients were retrospectively analyzed and correlated with the miRNA expression profiles. In the survival analysis, the mean of miR-106b level in adjacent non-tumor tissues was defined as the threshold (0.4 arbitrary unit from q-RT-PCR analysis). Samples with miR-106b expression levels higher than the threshold were classified into the "high-expression of miR-106b" group. Patients with levels lower than the threshold were classified into "low-expression of miR-106b" group. The overall and disease-free survival rates of patients were calculated using the Kaplan-Meier analysis.

Cells

Human hepatocellular carcinoma, Hep-3B 2.1-7 and Huh7 cells (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (DMEM, Hyclone) and minimum essential medium (MEM, Hyclone) containing 10% fetal bovine serum (FBS, GIBCO) and 100 IU of penicillin, 100 μg of streptomycin, and 0.25 μg of amphotericin B per milliliter, respectively. The cells were cultured in a humidified incubator with 5% CO_2 at 37 ℃.

RNA extraction and real-time RT-PCR

Total RNA was extracted using the RNeasy Plus Mini Kit (QIAGEN) according to the manufacturer's instructions. A total of 500 ng RNA was used to synthesize cDNA using a quantitative reverse transcription (RT) kit (Qiagen). The expression levels of miRNAs were analyzed using the TaqMan MicroRNA Assay Kit (Applied Biosystems) according to the manufacturer's instructions. The mRNA levels of human GAPDH and MCM7 were detected using the validated specific primers/probes of TaqMan Gene Expression Assays (Thermo Fisher Scientific) and the TaqMan Universal PCR Master Mix (Thermo Fisher Scientific). Real-time PCR assays were performed using the StepOne Real-Time PCR System (Applied Biosystems). The signals for miRNAs and inducible cellular MCM7 mRNAs were normalized to a small nuclear RNA, RUN48 and the mRNA signal of the housekeeping gene, human GAPDH.

Plasmid and transfection

The HBx protein expression plasmid was isolated and purified using the Plasmid Midi Kit (Qiagen). The Plasmid was transiently transfected into Hep-3B 2.1-7 and Huh7 cells using HyfectTM DNA transfection

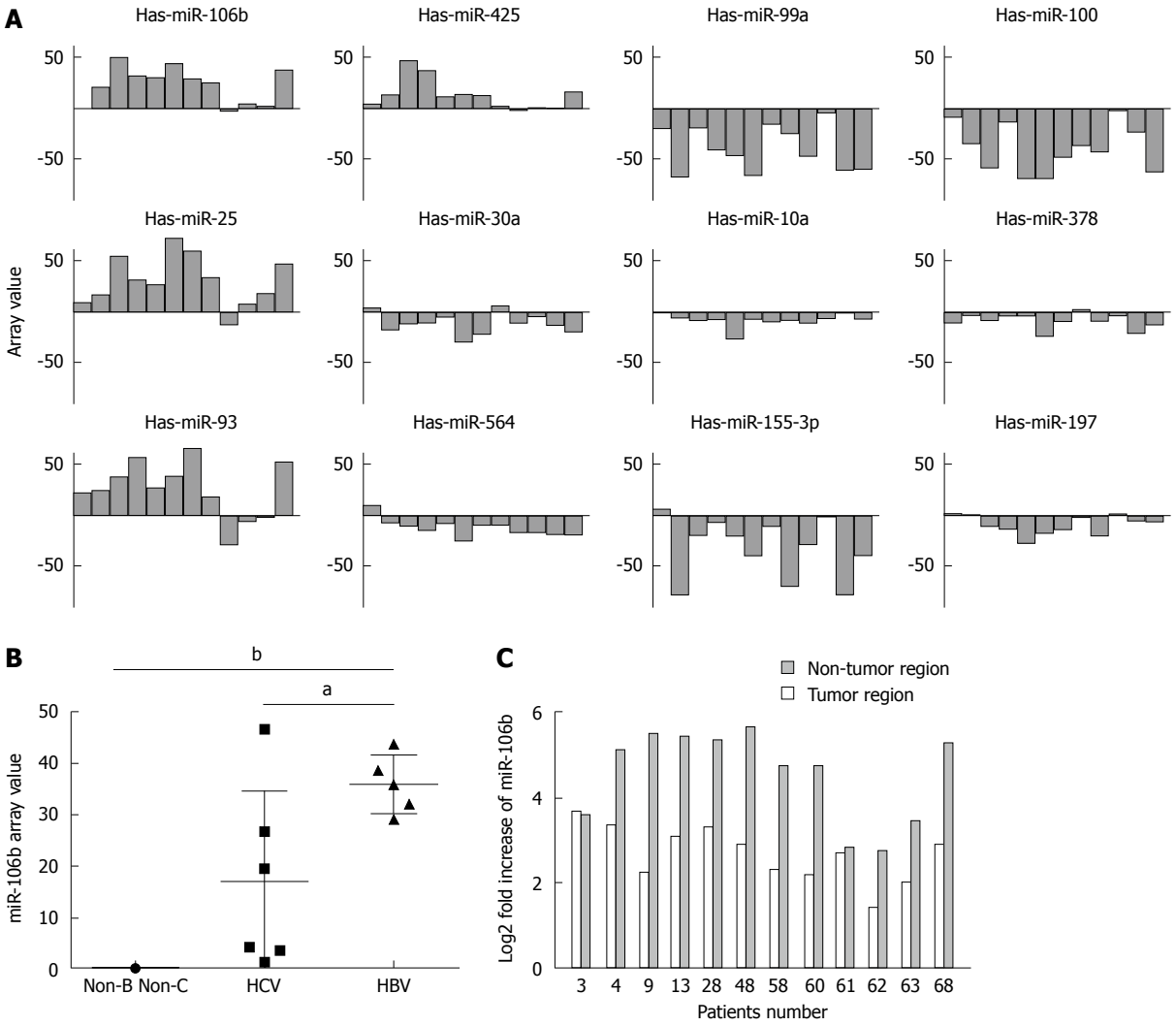

Figure 1 miRNA array analysis of the miRNA expression patterns in patients with distinct types of hepatocellular carcinoma (*n* = 12). A: The top 12 miRNAs significantly dysregulated in the tumor regions of hepatocellular carcinoma (HCC) patients based on statistical results ($P < 0.01$); B: miR-106b expression levels in tumor regions of patients with hepatitis B virus (HBV) -associated HCC, HCV-associated HCC, and non-B/non-C HCC; C: q-RT-PCR analysis of miR-106b expression values in the tumor and adjacent non-tumor regions of patients. Fold-increases were calculated after comparing the results with the miR-106b expression levels in normal liver samples. Data represent the mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.001$ *vs* HCV.

reagent (LEADGENE) according to the manufacturer's instructions. The expressions of HBx protein in the cell lines was confirmed using q-RT-PCR with HBx-specific primers/probe.

Statistical analysis

Statistical evaluation was completed using GraphPad Prism software version 5.01 (GraphPad, Inc., San Diego, CA, United States). The normal distribution of variables was assessed prior to selecting the tests to use for statistical analyses. The *W* value for the Shapiro-Wilk's method and the *D* value for the Kolomogorove method were used in the tests for normality. The values of miRNAs and MCM7 mRNA were analyzed using either the nonparametric one-way analysis of variance or unpaired *t* test, and the survival rates were analyzed using log rank analysis. The correlation between the patient outcomes and the miR-106b expression profiles were analyzed using Mann-Whitney *U* tests. The results are expressed as the mean ± SEM. A *P* value of less than 0.05 ($P < 0.05$, $P < 0.01$, $P < 0.001$) was considered significant.

RESULTS

miR-106b expression was up-regulated in HCC patients

Dysregulated miRNAs is a common characteristic of human tumors that could play an important role in oncogenesis or tumor suppression. To investigate the different miRNA expression profiles in HCC patients, we used miRNA array to analyze the miRNA expression patterns in 12 patients with distinct types of HCC including HBV-associated HCC, HCV-associated HCC, and non-B/non-C HCC in the 1st study cohort. The top 12 dysregulated miRNAs in HCC patients are listed in Figure 1A. miR-106b and the members of its associated cluster, miR-93 and miR-25 were up-regulated in HCC patients (Figure 1A). The value of

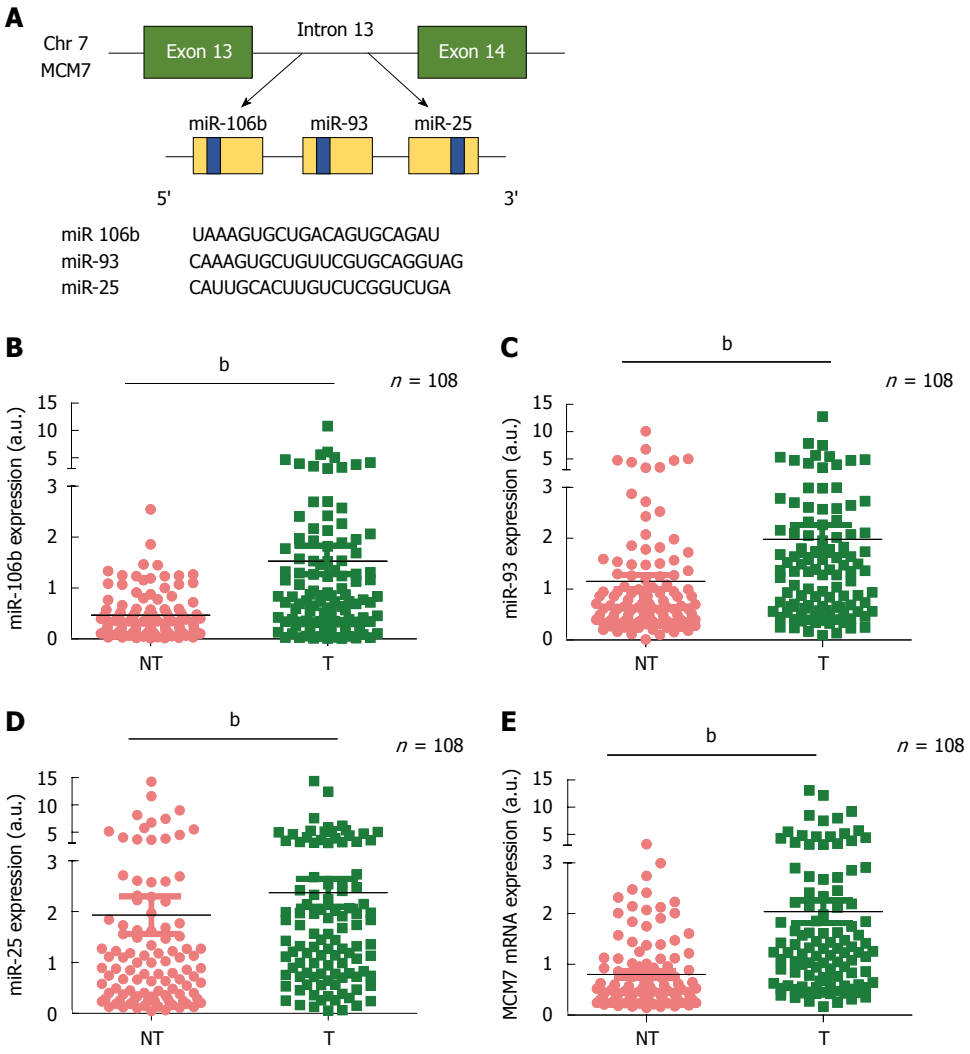

Figure 2 The mRNA expression levels of the miR-106b-25 cluster and MCM7 in tumor and non-tumor regions of hepatitis B virus-associated hepatocellular carcinoma patients (*n* = 108). A: Schematic representation of the miR-106b-25 cluster of miRNA (miR-106b, miR-93 and miR-25) within the 13th intron of the MCM7 gene. The yellow boxes represent pre-miRNAs. The blue boxes represent mature miRNAs; B-E: miR-106b (B), miR-93 (C), miR-25 (D), and MCM7 (E) expression levels in the tumor and non-tumor regions of hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC) patients, determined using q-RT-PCR. $^{b}P < 0.001$ NT *vs* T. a.u.: Arbitrary unit.

miR-106b expression in HBV-associated HCC patients was significantly higher than that in HCV- ($P < 0.05$) or non-B/non-C- ($P < 0.001$) associated HCC patients (Figure 1B). Furthermore, the levels of miR-106b expression in the 12 patients were confirmed using q-RT-PCR. In most cases, the values of miR-106b in the tumor regions were higher than in non-tumor regions (Figure 1C). These results indicated that miR-106b was significantly up-regulated in the tumor regions of HCC patients, particularly HBV-associated HCC patients.

miR-106b-25 cluster was co-transcribed with its host gene, MCM7 in HBV-associated HCC

miR-106b is located in an intergenic region embedded within intron 13 of the MCM7 gene in chromosome 7q22.1. This miRNA belongs to a cluster comprising miR-93 and miR-25 (Figure 2A). To determine whether the miR-106b promoter transcribes the associated gene or this gene is co-transcribed with the host gene, MCM7, Mass array EpiTyper was performed to detect the methylation landscape of MCM7 in the 12 patients. The results demonstrated that only the promoter and 3'-UTR of MCM7 could be detected within the methylation landscape suggesting that miR-106b is also co-transcribed with its host gene, MCM7 in HCC (data not shown). To further confirm whether the expression of miR-106b is higher in the tumor tissues of HBV-associated HCC patients, we expanded the sample size to 108 patients and validated the miRNA levels through q-RT-PCR in the 2nd study cohort. The results showed that the miR-106b levels were significantly higher in tumor tissues compared with normal tissues ($P < 0.001$) and this phenomenon was observed in more than 70% of HBV-associated HCC patients (Figure 2B). In addition, the expression of miR-93 and miR-25 was significantly up-regulated in the tumor tissues of HBV-associated HCC patients ($P <$

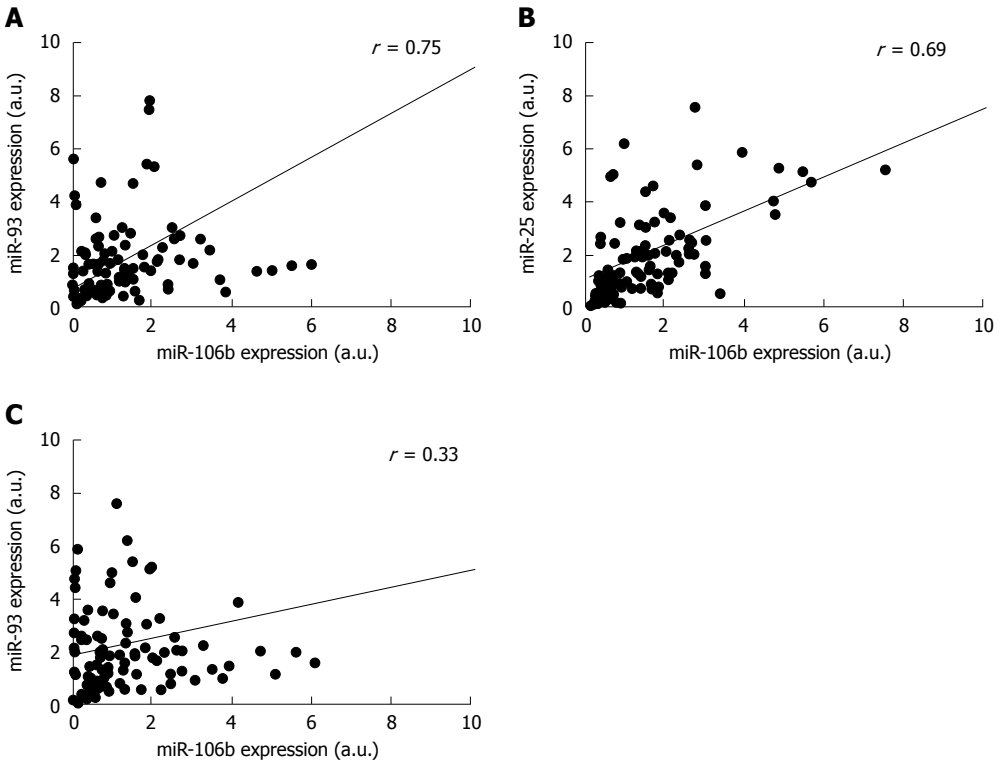

Figure 3 The correlation analysis miR-106b *vs* miR-93, miR-106b *vs* miR-25, and miR-93 *vs* miR-25 expression in tumor regions of patients with hepatitis B virus-associated hepatocellular carcinoma (*n* = 108). A: The correlation between miR-106b and miR-93 expression; B: The correlation between miR-106b and miR-25 expression; C: The correlation between miR-93 and miR-25 expression. Data represent the correlation coefficient (r). a.u.: Arbitrary unit. HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus.

0.001) (Figure 2C and D). The mRNA expression level of the MCM7 gene was also significantly increased in tumor regions compared with the adjacent non-tumor regions ($P < 0.001$) (Figure 2E). Furthermore, the expression of miR-106b, miR-93 and miR-25 showed a positive correlation in HBV-associated HCC tissues (miR-106 *vs* miR-93, $r = 0.75$; miR-93 *vs* miR-25, $r = 0.69$; miR-106b *vs* miR-25, $r = 0.33$) (Figure 3A-C). These results indicated that the miR106b-25 cluster is up-regulated in the tumor regions and co-transcribed with its host gene, MCM7 in HBV-associated HCC.

Up-regulation of miR-106b expression corresponds with decreased survival time in HBV-associated HCC patients

We further evaluated the relationship between the miR-106b expression and clinical outcomes in HBV-associated HCC patients; the patients were divided into two groups, high miR-106b expression and low miR-106b expression and the overall and disease-free survival rates in these two groups were analyzed. The data showed a negative correlation between the miR-106b expression level and survival time of HBV-associated HCC patients (Figure 4A and B). Relatively poor overall and disease-free survival rates were observed for the individuals in the high miR-106b expression group (overall survival in the 5th year: 65%; disease-free survival in the 5th year: 40%) compared with the low miR-106b expression group (overall survival in the 5th year: 93%; disease-free survival in the 5th year: 57%) ($P < 0.05$). These results demonstrated that poor prognosis is correlated with HBV-associated HCC patients with higher miR-106b expression.

miR-106b expression levels are correlated with HCC differentiation

The demographic and clinical features of patients were retrospectively analyzed and correlated with the miR-106b expression profiles to determine the specific features associated with miR-106b expression. HCC differentiation but not underlying liver disease, microvascular invasion, tumor number, tumor size, recurrence after surgery, and pathological staining were significantly correlated with miR-106b expression (Table 2). The miR-106b expression level in patients with well HCC differentiation (2.24 ± 0.44 a.u.) was significantly lower than that in patients with moderate (5.32 ± 1.00 a.u.) and poor HCC differentiation (4.85 ± 1.02 a.u.) (well *vs* moderate, P=0.0359; well *vs* poor, P=0.0145). These results indicated that low levels of miR-106b expression result in well HCC differentiation.

HBx promotes miR-106b expression in HCC cells

Next, we investigated the mechanism of how miR-106b expression is regulated in HBV-associated HCC. HBx protein is necessary for HBV replication and acts as a trans-activator for the modulation of the signaling

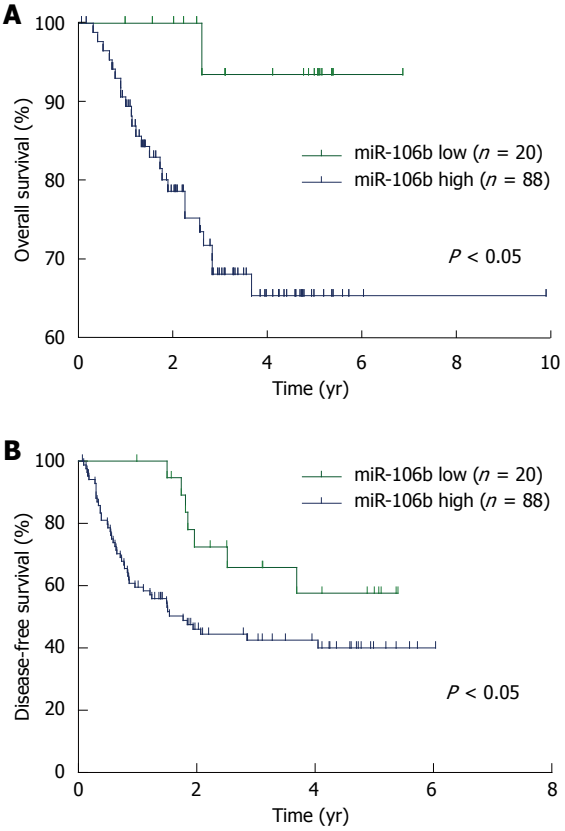

Figure 4 Kaplan-Meier overall and disease-free survival curve of patients with hepatitis B virus-associated hepatocellular carcinoma based on miR-106b expression (*n* = 108). Hepatitis B virus (HBV)-associated hepatocellular carcinoma (HCC) patients were divided into two groups including miR-106b low (*n* = 20) and miR-106b high (*n* = 88) groups based on the expression levels of miR-106b. A: Overall survival curve; B: Disease-free survival curve. Log-rank test was used for statistical analysis. $P < 0.05$ was considered significant.

Table 2 Correlation analysis of clinical outcomes with miR-106b expression profiles (*n* = 108)[1]

	Patient numbers *n* (%)	*P* value	Significant[2]
Underlying liver disease		0.374	NS
Liver cirrhosis	39 (36)		
Non-cirrhosis	69 (64)		
HCC differentiation			
Well *vs* moderate	24 (22) *vs* 74 (69)	0.036	[a]
Well *vs* poor	24 (22) *vs* 9 (8)	0.015	[a]
Moderate *vs* poor	74 (69) *vs* 9 (8)	0.238	NS
Unknown	1		
Microvascular invasion		0.856	NS
Yes	30 (28)		
No	78 (72)		
Tumor number		0.798	NS
Single tumor	87 (81)		
> 1 tumor	21 (19)		
Tumor size (cm)[3]		0.812	NS
< 5	72 (67)		
⩾ 5	36 (33)		
Recurrence after surgery		0.687	NS
Yes	58 (54)		
No	50 (46)		
Pathological staging			
Stage Ⅰ *vs* stage Ⅱ	39 (36) *vs* 51 (47)	0.968	NS
Stage Ⅰ *vs* stage Ⅲ	39 (36) *vs* 18 (17)	0.625	NS
Stage Ⅱ and stage Ⅲ	51 (47) *vs* 18 (17)	0.575	NS
		Total patients, *n* = 108	

[1]The patient characteristics are summarized based on the clinical outcomes. Demographic and clinical features of patients were retrospectively analyzed and correlated with the miR-106b expression profiles; [2]The correlation between miR-106b expression values and the patient numbers of each clinical outcome parameter were analyzed using Mann-Whitney *U* tests. [a]$P < 0.05$ was considered significant; [3]Tumor size represents the maximum diameter of the tumor nodule. The diameter of the largest nodule was 16 cm. NS: No significant difference; HCC: Hepatocellular carcinoma.

pathways involved in the HBV replication and HCC development. To determine whether HBx protein contributes to the regulation of miR-106b expression in HBV-associated HCC, we used an HBx protein over-expression system. Huh7 and Hep 3B cells were transiently transfected with an HBx-expression plasmid, and subsequently the expression levels of the miR-106b-25 cluster and MCM7 were analyzed. The levels of miR-106b, miR-93, and miR-25 were significantly increased (all $P < 0.05$), peaking at 6 h post transfection in both Huh7 and Hep 3B cells (Figure 5A and B). The MCM7 mRNA levels were also gradually increased after 6 h post transfection compared with the un-transfected control group in both Huh7 ($P < 0.01$) and Hep 3B ($P < 0.05$) cells (Figure 5C and D). These results suggested that the HBx protein might contribute to the up-regulation of the miR-106b-25 cluster and MCM7 in HBV-associated HCC.

DISCUSSION

HCC ranks as the third leading cause of cancer-related deaths worldwide with increasing cases in many countries[1,27]. Increasing evidence has shown that many miRNAs are dysregulated in HCC and play a crucial role in the development of HCC by affecting cell proliferation, apoptosis, migration, *etc*[28]. Therefore, the identification of a key miRNA associated with tumor progression in HCC could provide an accurate marker for diagnosis and a new direction for a novel therapeutic approach. In the present study, we found that miR-106b plays an important role in tumor progression in HBV-associated HCC.

Recent studies have demonstrated that several miRNAs are involved in the life cycle and infectious processes of HBV and HBV-associated liver diseases, including fibrosis, cirrhosis and HCC[29,30]. Herein, we observed the up-regulation of the miR-106b-25 cluster in HCC, particularly in HBV-associated HCC. The expression of miR-106b, miR-93 and miR-25 was positively correlated in HBV-associated HCC tissues, consistent with the results of previous study showing a similar correction pattern in gastric cancer, suggesting the co-transcription of miR-106b-25 in biosynthesis[31]. Interestingly, the correlation coefficients between each other were not consistent, indicating that additional mechanisms might be involved in the regulation of miRNA expression. Notably, the up-regulation of

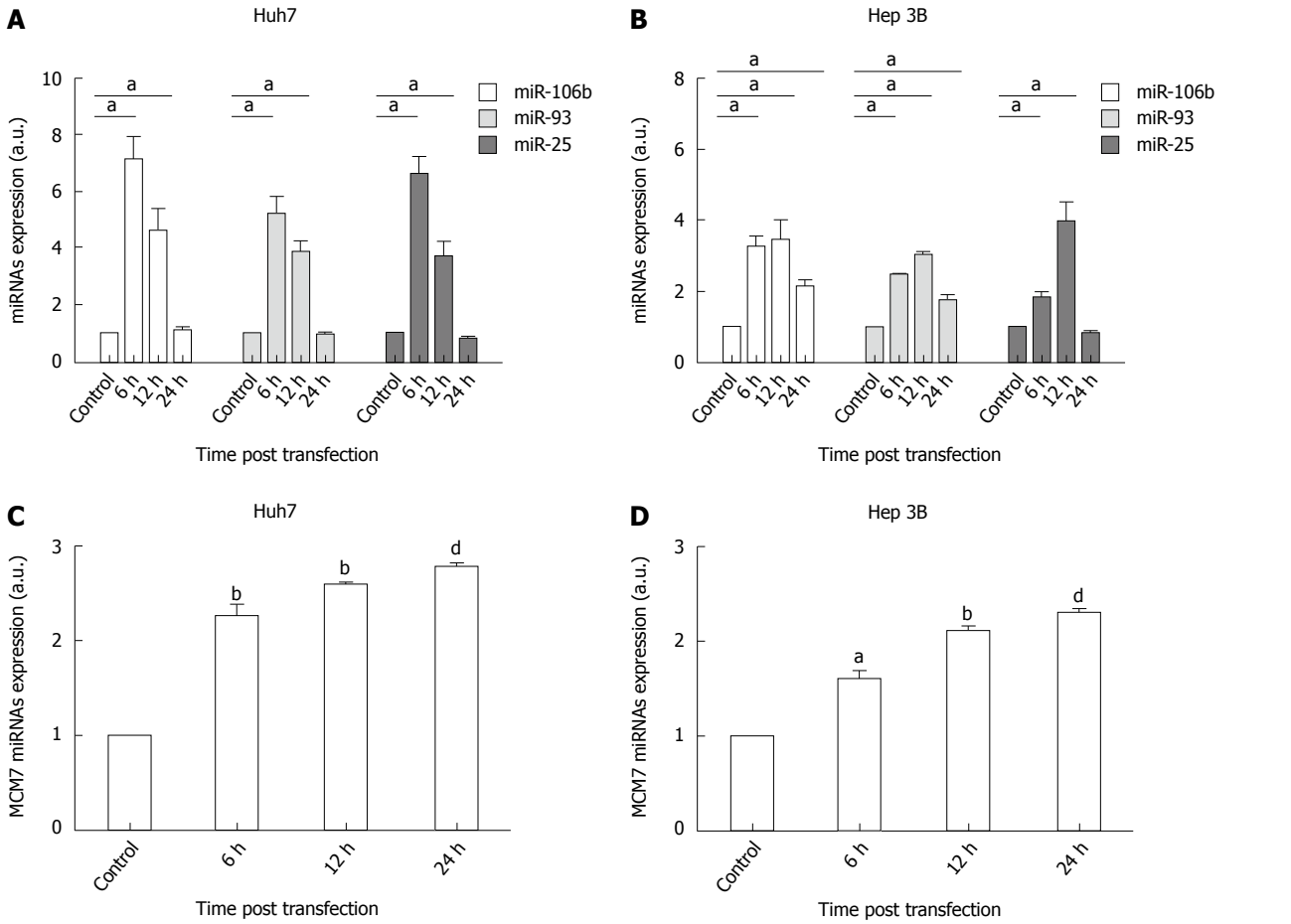

Figure 5 The mRNA expression levels of miR-106b-25 cluster and MCM7 in hepatitis B virus X protein-transfected hepatocellular carcinoma cell lines (*n* = 3). The hepatitis B virus X protein (HBx) protein expression plasmid was transiently transfected into Huh7 and Hep-3B cells. miR-106b, miR-93, miR-25, and MCM7 expression levels at 0 (control), 6, 12, and 24 h post transfection were detected using q-RT-PCR. A: miRNAs expression levels in HBx-transfected Huh7 cells; B: miRNAs expression levels in HBx-transfected Hep-3B cells; C: MCM7 expression levels in HBx-transfected Huh7 cells; D: MCM7 expression levels in HBx-transfected Hep-3B cells. Data represent the mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{d}P < 0.001$ *vs* control. a.u.: Arbitrary unit. HCC: Hepatocellular carcinoma.

miR-106b but not miR-93 and miR-25 expression corresponds to decreased survival times, increased recurrence rates and HCC differentiation in HBV-associated HCC patients (data not shown). This effect might reflect the different biological functions of individual miRNAs. Previous studies have indicated that miR-106b and miR-93 directly target the cell-cycle inhibitor, CDKN1A (p21), and miR-25 inhibits cell apoptosis through the down-regulation of a the pro-apoptotic gene, *BCL2L11* (Bim), in gastric cancer[31]. Other studies have shown that miR-106b is not only involved in cell cycle inhibition but might also play an anti-apoptosis role in cancer cells[32]. The data obtained in the present study, support the idea that miR-106b has a greater effect on promoting tumor progression compared with the other members in the same cluster.

However, HBV-associated protein regulates the expression of several miRNAs to assist with viral replication and survival and HCC development[33,34]. In a previous study, we showed that HBx protein up-regulates mTOR signaling through IKKβ to increase cell proliferation and VEGF production in HCC[35]. Furthermore, HBx protein is highly expressed in the cytoplasm of hepatocytes after HBV infection, thereby promoting tumorigenesis through the induction of mitochondrial dysfunction, involving several signaling pathways associated with tumorigenesis through the regulation of non-coding RNAs (ncRNAs) and epigenetic changes[8,34,36]. The results of present study showed that HBx over-expression promoted the transcription of miR-106 in HCC cell lines. This finding might explain why miR-106b was remarkably up-regulated in HBV-associated HCC but not in other types of HCC. However, the precise regulatory mechanism of HBx in miR-106b expression should be further investigated.

Based on these results, the miR-106b-25 cluster was co-transcribed with its host gene, MCM7 in HBV-associated HCC. Previous studies have indicated that MCM proteins are involved in critical steps of DNA synthesis[37]. MCM proteins bind to DNA replication origins during the initiation step, and subsequently the MCM proteins provide the helicase activity to unwind the template DNA ahead of the fork for DNA elongation. In primary gastric tumors and normal mucosa, the mRNA expression of MCM7 is precisely

correlated with the expression of the miR-106b-25 cluster[18]. The detailed regulatory mechanism between miR-106b-25 and MCM7 and whether MCM7 is involved in the miR-106b-mediated influence on HBV-associated HCC need to be further examined.

In conclusion, the results of present study indicate that miR-106b is up-regulated and co-transcribed with its host gene MCM7 in HBV-associated HCC. The up-regulation of miR-106b expression corresponds with a decrease in survival time, and an increase in the recurrence rate and HCC differentiation in HBV-associated HCC patients. Furthermore, HBx over-expression increased the RNA levels of the miR-106b-25 cluster and MCM7 in human hepatocellular carcinoma cell lines. These results suggest that HBx enhances the transcription of miR-106b to promote tumor progression in HBV-associated HCC. These findings provide a potential diagnostic marker and therapeutic target for HBV-associated HCC.

COMMENTS

Background

MicroRNAs (miRNAs) are involved in the progression of numerous types of cancers. Chronic hepatitis B virus (HBV) infection is one of the major risks for hepatocellular carcinoma (HCC), and through the regulation of miRNA expression, the virus promotes carcinogenesis in HCC.

Research frontiers

HBV infection not only induces liver inflammation but also produces viral oncoproteins to influence HCC progression. Previous studies have reported that many miRNAs are dysregulated in the HBV-associated HCC. However, the role of miRNA in tumor progression and regulation remains unclear. In the present study, the authors report that the hepatitis B virus X protein (HBx) protein enhances miR-106b expression to promote HCC progression.

Innovations and breakthroughs

Previous studies regarding the role of miRNA in tumors are limited in the use of correlation analyses and use small-scale cohort studies to address this issue. In addition, the function of the HBx protein in HCC progression is controversial. This work represents the first large-scale cohort study demonstrating that the miR-106b-25 cluster and its host gene, MCM7, are overexpressed in HBV-associated HCC. The results also suggest that the HBx protein enhances miR-106b transcription to promote tumor progression.

Applications

Because miR-106b is up-regulated in patients with HBV-associated HCC and correlates with poor disease outcome, these finding could provide a novel diagnostic marker and a therapeutic target for HBV-associated HCC.

Peer-review

In this manuscript the authors investigated the effect of miR-106b on tumor progression in HBV-associated HCC in a clinical model. This study provides evidence that enhanced transcription of miR-106b with its host gene MCM7 in HBV-associated HCC is associated with tumor progression and poor outcome. This study is well designed and the results are acceptable to draw the conclusions stated herein.

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ORIGINAL ARTICLE

Basic Study

Chitooligosaccharides promote radiosensitivity in colon cancer line SW480

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Abstract

AIM: To investigate the anti-proliferation and radiosensitization effect of chitooligosaccharides (COS) on human colon cancer cell line SW480.

METHODS: SW480 cells were treated with 0, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/mL of COS for 48 h. CCK-8 assay was employed to obtain the cell survival ratio of SW480 cells, and the anti-proliferation curve was observed with the inhibition ratio of COS on SW480 cells. The RAY + COS group was treated with 1.0 mg/mL of COS for 48 h, while both the RAY and RAY+COS groups were exposed to X-ray at 0, 1, 2, 4, 6 and 8 Gy, respectively. Clonogenic assay was used to analyze cell viability in the two groups at 10 d after treatment, and a cell survival curve was used to analyze the sensitization ratio of COS. The RAY group was exposed to X-ray at 6 Gy, while the RAY+COS group was treated with 1.0 mg/mL of COS for 48 h in advance and exposed to X-ray at 6 Gy. Flow cytometry was employed to detect cell cycle and apoptosis rate in the non-treatment group, as well as in the RAY and RAY + COS groups after 24 h of treatment.

RESULTS: COS inhibited the proliferation of SW480 cells, and the inhibition rate positively correlated with the concentration of COS ($P < 0.01$). Cell viability decreased as radiation dose increased in the RAY and RAY+COS groups ($P < 0.01$). Cell viabilities in the RAY+COS group were lower than in the RAY group at all doses of X-ray exposure ($P < 0.01$), and the sensitization ratio of COS on SW480 cells was 1.39. Compared with the non-treatment group, there was a significant increase in apoptosis rate in both the RAY

and RAY + COS groups; while the apoptosis rate in the RAY+COS group was significantly higher than in the RAY group ($P < 0.01$). In comparing these three groups, the percentage of G_2/M phase in both the RAY and RAY + COS groups significantly increased, and the percentage of the S phase and G_0/G_1 phase was downregulated. Furthermore, the percentage in the G_2/M phase was higher, and the percentage in the S phase and G_0/G_1 phase was lower in the RAY + COS group *vs* RAY group ($P < 0.01$).

CONCLUSION: COS can inhibit the proliferation of SW480 cells and enhance the radiosensitization of SW480 cells, inducing apoptosis and G_2/M phase arrest.

Key words: Chitooligosaccharides; Cancer of colon; Radiotherapy; Radiosensitization; Apoptosis; Cell cycle

Core tip: In this study, the colorectal cancer cell line SW480 that is homologous with colon-rectum was used as the research tool. It was confirmed that chitooligosaccharides (COS) not only directly blocked SW480 cell proliferation, but also enhanced radiotherapy effects. Furthermore, COS induced a large amount of SW480 cell apoptosis, and induced a large number of cells to remain in the G2/M phase with radiation-sensitive killing effect. Thus, the sensitivity of SW480 cells to radiation was effectively enhanced 1.39 times. This is beneficial for the therapeutic effect.

Han FS, Yang SJ, Lin MB, Chen YQ, Yang P, Xu JM. Chitooligosaccharides promote radiosensitivity in colon cancer line SW480. *World J Gastroenterol* 2016; 22(22): 5193-5200 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i22/5193.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i22.5193

INTRODUCTION

According to the latest Chinese cancer epidemic profile survey, the 2010 cancer morbidity and mortality rates in China were 235.23/100000 and 148.81/100000, respectively. Among these cancers, colorectal cancer incidence and mortality have shown an obvious predominantly evident upward trend again, causing this to be the focus of medical experts[1-4]. In China, colorectal cancer has a prevalence of 16.14%, allowing a leap up to fifth place among cancers; and the incidence in males is as high as 18.75%. In 2012, over 250000 colorectal cancer cases were added nationwide; this total accounts for 18.6% in the world[5,6]. China has become a big focus at the forefront of colorectal cancer research, since efficient and low toxicity treatments are needed. Since the discovery of radiation, radiotherapy has lasted for centuries as the main indispensable weapon against cancer that is active in clinic. Statistics have shown that more than 70% and 50% of cancer patients are in need of this kind of therapy in China and the United States, respectively[7,8]. Although radiotherapy has great significance for cancer treatment, killing cancer cells could injure healthy tissues, causing malignant complications. This has been a researcher's hurdle that is difficult to bypass. However, radiosensitizers then emerged as a necessity of the times. Chitooligosaccharides (COS) are products of chitin, having good solubility and a high absorption rate, making the ratio of the carbohydrate polymer more advantageous for biological applications; and they have become the new favorite of medical researchers[9,10]. Although there have been many reports about COS anticancer effects[11-14], there are few studies on the applications of radiation sensitization. In this study, human colon cancer SW480 cells were selected and treated with COS application in parallel with radiation, to verify that COS can enhance radio sensitivity for colorectal cancer cell line; this is reported below. We expect to further explore this superior and low-damage anticancer therapies.

MATERIALS AND METHODS

Seven COS concentration levels were established and 3-hole samples were simultaneously cultured in parallel with each level SW480 cells were subcultured to the logarithmic phase (human colon cancer cells SW480; Shanghai Cell Institute of Chinese Academy of Sciences). After digestion, the cells were diluted to a concentration of 5×10^4 cells/mL according to the 0.1 mL/hole access in a 96-well plate. A suitable environment was set (CO_2 incubator, Shanghai Gemtop Scientific Instrument CO.,Ltd.) for adherent growth, and diluted COS (Chitooligosaccharides, Shanghai Huich Biotech Inc.) was replaced after 24 h with fresh medium at 0.11 mL/hole, and added into each well at COS concentrations of 0, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 mg/mL After 48 h of COS application, CCK-8 reagent (CCK-8 Kit, Shanghai Liruibio Technology Co., Ltd.) was added along the pore walls at 0.01 mL/hole. Then, the culture plate was tapped to mix reagent and culture medium. After four hours of sufficient reaction, OD absorbance at $\lambda = 450$ nm was detected at all levels. The experiment was repeated three times to investigate the inhibitory effect of COS on SW480 cell proliferation. Accordingly, 1.0 mg/mL of COS concentration was selected for follow-up studies.

Six X-ray dose levels were established and levels were divided into the RAY group and RAY+COS group; each group was simultaneously cultured in parallel with the 3-hole sample. According to 0-, 1- and 2-Gy dose levels at 200/well, 4- and 6-Gy dose levels at 400/well, and 8-Gy dose level at 800/well inoculation amounts, respectively, different concentrations of single-cell suspensions in 6-well culture plate were set in an incubator with a suitable environment for growth

Table 1 Inhibition of chitooligosaccharides for the proliferation of SW480 cells

COS concentration (mg/mL)	OD (mean ± SD)	Inhibition rate
0	1.019 ± 0.007	-
0.5	0.969 ± 0.005[1]	4.91%
1.0	0.908 ± 0.008[2]	10.89%
2.0	0.804 ± 0.006[3]	21.10%
3.0	0.692 ± 0.007[4]	32.09%
4.0	0.580 ± 0.006[5]	43.08%
5.0	0.433 ± 0.008[6]	57.51%
P	0	-
F	137.6	-

Compared with COS concentrations in the 0 mg/mL Group: [1]P = 0.000, q = 17.437; [2]P = 0.000, q = 31.326; [3]P = 0.000, q = 80.047; [4]P = 0.000, q = 99.096; [5]P = 0.000, q = 142.849; [6]P = 0.000, q = 165.379. COS: Chitooligosaccharides.

adherence. After six hours, appropriate amounts of COS were added into each well to reach a 1.0 mg/mL concentration in the RAY + COS group, while equal amounts of infiltrating medium were added into each hole and cultured for 48 h in the RAY group. Both groups were stamped with 1-cm thick tissue analogs in the culture plates and X-ray irradiated (Electron linear accelerator, Nanjing Chuang Rui Ying Biotechnology Co., Ltd.) at a distance of 100 cm with a dose rate of 2 Gy/min. Incubation continued for 10 d, the cells were washed, fixed and stained again; then, the number of cells were counted as 50 or more units of cell clusters. The experiment was repeated three times for statistical data, and the cell survival curve was draw up from the final slope of the D_0 value obtained by sensitizing ratio SER = $D_{0\,(RAY)}/D_{0\,(RAY+COS)}$.

The three groups were established simultaneously in parallel with the 3-hole samples. The concentration of 1 × 10^5/mL cell suspension was inoculated into 6-well culture plate with a suitable growth-adherent environment for 24 h. In the RAY + COS group, the correct amount of COS was added to reach a 1.0 mg/mL concentration. In the non-treatment group and RAY group, equal amounts of medium were added. In the RAY group and RAY + COS group, infiltration was carried out for 48 h and samples were exposed to 6-Gy X-ray irradiation. After replacing with fresh medium, cells were cultured for 48 h. After digestion, rinsing, dilution and other treatments were performed to determine the cell cycle stage and extent of apoptosis in each group.

Statistical analysis

Using SPSS19.0 statistical software for statistical analysis, OD value, survival rate, apoptosis rate and cell cycle distribution ratios were presented as mean ± SD. OD values for each COS concentration level and cell survival rates at X-ray dose levels were compared among multiple groups using ANOVA analysis and compared between the two groups using SNK-q test. For the RAY group and RAY + COS group, cell survival rates under different X-ray doses, cell cycle control experiments among the three groups, and apoptosis rates between the three groups were compared by *t*-test. $P < 0.05$ was considered statistically significant.

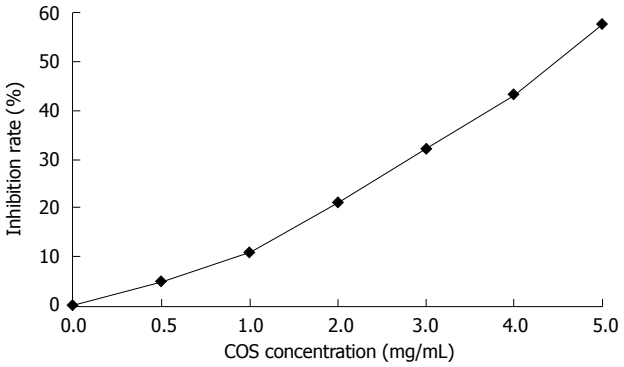

Figure 1 Inhibition curve of chitooligosaccharides for proliferation of SW480 cells.

RESULTS

Inhibitory effect of COS on SW480 cell proliferation

In comparing the COS concentration of the OD value in the 0 mg/mL group, OD values progressively reduced at all levels as COS concentration increased, and the difference was statistically significant ($P < 0.01$). After 48 h of treatment with 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 μmg/mL of COS concentration, SW480 cell survival rates were significantly lower than in the negative control group; and the differences were statistically significant ($P < 0.01$). The inhibition rate and the concentration of COS showed a positive correlation (Table 1, Figure 1).

Comparison of effects of different radiation dose on cell survival rate between groups

With an irradiation dose of 0 Gy as a reference standard, survival rate in the RAY and RAY + COS groups progressively reduced with increased radiation dose, and the difference was statistically significant ($P < 0.01$). The reduction in cell survival rate was greatest in the RAY + COS group. At dose levels 1, 2, 4, 6 and 8 Gy, survival rate in the RAY+COS group was significantly lower than in the RAY group, and the difference was statistically significant ($P < 0.01$). SER was 1.39 for COS in SW480 cells (Table 2, Figure 2).

Cell apoptosis rate between groups

Compared with proliferation in the non-treatment group, apoptosis rate in the RAY and RAY + COS groups increased sharply, the differences were statistically significant ($P < 0.01$). Apoptosis rate in the RAY+COS group also revealed a significant increase compared with the RAY group, and the difference was statistically significant ($P < 0.01$; Table 3, Figure 3).

Comparison of cell cycle distribution between groups

Compared with the non-treatment group, the proportion in G2/M phase in the RAY and RAY + COS

Table 2 Comparison of cells survival rates for different radiation doses between the RAY and RAY + COS group

Irradiation dose (Gy)	Cell survival rate (%)	
	RAY	RAY + COS
0	99.22 ± 3.51	99.17 ± 4.06[1]
1	90.67 ± 3.82	85.30 ± 3.38[2]
2	73.69 ± 3.45	56.11 ± 2.95[3]
4	45.95 ± 3.41	28.64 ± 2.76[4]
6	23.84 ± 2.20	12.53 ± 2.03[5]
8	8.68 ± 1.75	3.81 ± 1.16[6]
P	0	0
F	182.7	243.2

Compared with RAY group: [1]P = 0.978, t = 0.028; [2]P = 0.006, t = 3.158, [3]P = 0.000, t = 11.619; [4]P = 0.000, t = 11.837; [5]P = 0.000, t = 11.335; [6]P = 0.000, t = 6.959. COS: Chitooligosaccharides.

Table 3 Comparison of cell apoptosis rates among the three groups

Groups	Cell apoptosis rate
Non-treatment group	1.79 ± 0.37
RAY group	9.33 ± 1.05[1]
RAY + COS group	22.64 ± 1.27[2,3]

Compared with the control group: [1]P = 0.000, t = -20.318; [2]P = 0.000, t = -47.286; compared with the RAY Group: [3]P = 0.000, t = -24.232. COS: Chitooligosaccharides.

Table 4 Comparison of cell cycle distribution among the three groups

Groups	S (%)	G0/G1 (%)	G2/M (%)
Non-treatment group	30.15 ± 0.82	39.41 ± 1.05	30.44 ± 0.68
RAY group	22.48 ± 0.74[1]	30.51 ± 0.86[2]	47.01 ± 0.52[3]
RAY + COS group	18.89 ± 0.65[4,7]	24.34 ± 0.46[5,8]	56.77 ± 0.28[6,9]

Compared with the control group: [1]P = 0.000, t = 20.832; [2]P = 0.000, t = 19.672; [3]P = 0.000, t = -58.070; [4]P = 0.000, t = 32.283; [5]P = 0.000, t = 39.438; [6]P = 0.000, t = -107.412; compared with the RAY group: [7]P = 0.000, t = 10.935; [8]P = 0.000, t = 18.979; [9]P = 0.000, t = -49.577. COS: Chitooligosaccharides.

groups significantly increased, while the proportions in S and G0/G1 phase all reduced; the differences were statistically significant (P < 0.01). Compared with the RAY group, the proportions in S phase and G0/G1 phase in the RAY + COS group were smaller, while the G2/M phase was significantly longer; the differences were statistically significant (P < 0.01). See Table 4, Figure 4.

DISCUSSION

Rapid economic growth gives rise to the rapid development of science and technology. However, improvements in medical technology have failed in stopping cancer from affecting human health. Modern unhealthy diets and living habits stimulate and mainly cause the continuous rise in colorectal cancer

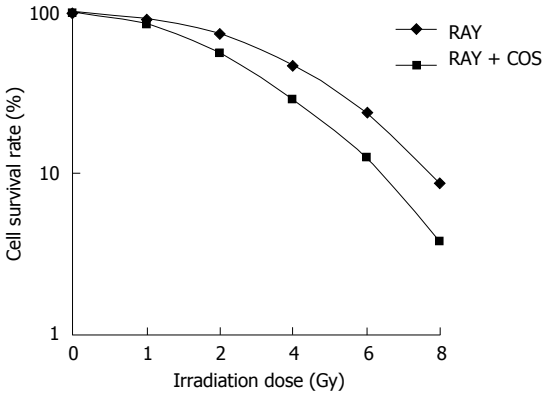

Figure 2 Cells survival rates at different radiation doses between the RAY and RAY + COS groups. COS: Chitooligosaccharides.

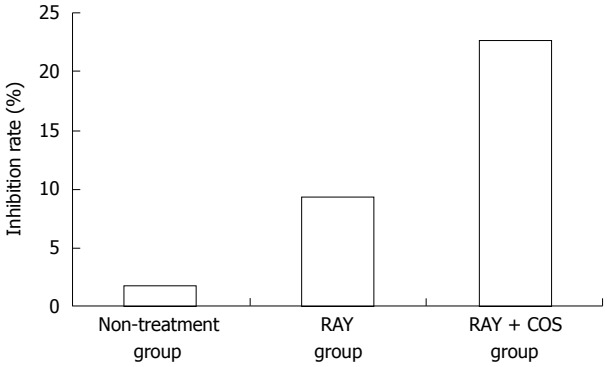

Figure 3 Cell apoptosis comparison between the three groups.

morbidity[15-17]. Statistical data from international cancer research institutions have indicated that China's 2012 annual new-onset cases of colorectal cancer reached 1.47 times that of cases in 2006, and the total incidence grew by nearly 50% in six years[18]. Regarding the incidence of colorectal cancer in China, the annual growth rate rose sharply to more than two times the international average[19]. This situation is not optimistic, and the exploration of more effective drugs and treatment without delay is of great significance.

Inhibitory effect of COS on proliferation of colon cancer SW480 cells

Derived from the depolymerization of chitosan, COS has been considered as the human healthy "almighty" guardian by the biomedical field. It can improve body acid-base balance, activate immune function, remove blood lipids, lower blood sugar, and regulate a variety of physiological activity[20-22]. Particularly, it has anti-tumor effects, which have been a research focus for domestic and foreign scholars in recent years. Based on historical reports, COS has a widespread growth-blocking effect on HL-60, RBL-2H3, SGC-7901 and tumor cell lines of other organs, and there are a variety of ways to achieve this effect[23-25]. In this study, the colorectal homologous colorectal SW480 cell line was the target. This study confirms that COS directly

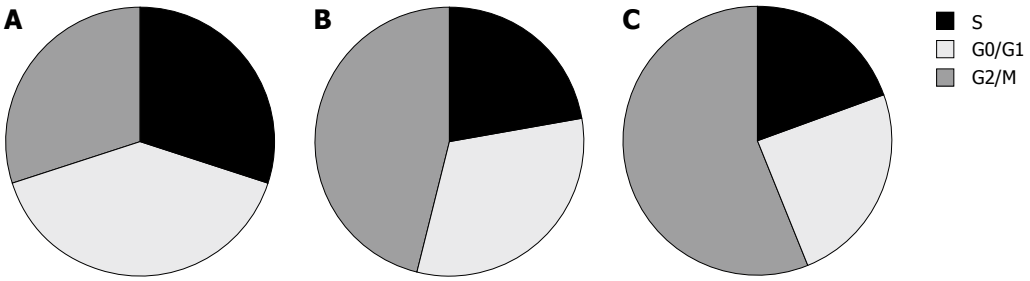

Figure 4 Comparison of cell cycle distribution among the three groups. A: Non-treatment group; B: RAY group; C: RAY + COS group. COS: Chitooligosaccharides.

blocks SW480 cell proliferation, and its inhibitory effect increases with the concentration of COS application showing increase in increments; 5 mg/mL of COS treatment for 48 h induced SW480 cell viability to decrease by 57.51%.

COS enhances SW480 cell sensitivity to radiation

Radiotherapy has an irreplaceable position in the treatment of cancer. It is widely used in various stages of the disease course; preoperatively it shrinks tumors to create conditions for radical enterectomy, it removes residues postoperatively to prevent recurrence, and it cooperates with chemotherapy to prevent metastasis. Early radiotherapy for nasopharyngeal carcinoma, skin cancer and cervical cancer has a possibility of more than 9% cure[26-28]. Early exposure to radiotherapy also can improve the 5-year survival rate from 70% to 80% for esophagus and prostate cancer[29,30]. Although radiotherapy has significantly prevents pain in patients with organ disease, there are also shortcomings. Radiation attack precision is limited, and often implicates normal tissue surrounding lesions, leading to tissue damage, which adds to the double burden of cancer patients both physically and mentally. This study found that X-rays have a mass destruction effect on SW480 cells, and cell death increased with radiation dose; while before radiotherapy, COS application obviously makes the cell survival rate decrease, COS can effectively increase SW480 cell sensitivity to radiation by 1.39 times. Furthermore, this controlled experiment has shown that the RAY + COS group had significantly improved treatment efficacy with the increase in apoptosis rate, and the cell cycle distribution changed significantly.

COS achieves radiosensitization by promoting SW480 cell apoptosis

Apoptosis maintains homeostasis in the body. However, it has an independent regulatory program that takes the initiative to open the "die" mode to conserve limited resources when cells are faced with adverse living conditions. Tumor cells lose this order of regulation and fall into a disordered and uncontrolled proliferation cycle. Radiation-induced apoptosis has been demonstrated for a long time[31-34]. COS combined with radiotherapy reverses the deactivation of the mechanism of apoptosis in a cancer cell to a greater degree, and pulls it back to its normal life trajectory. The complexity of the entire process of cell apoptosis involves cooperation of multiple genes and proteins. Krysko's research has indicated that COS can activate apoptosis promoter Caspase-3[35,36]. Tan believes that COS can damage mitochondrial membrane stability and release Cyt C into the cytosol[37]. Mates has also reported that COS can down-regulate environmental GSH activity and stimulate oxidative damage[38,39]. A number of conclusions have confirmed that COS has a positive effect on the apoptosis of tumor cells.

COS achieves radiosensitization by changing the SW480 cell cycle distribution

The mechanism of action of radiotherapy is to destroy DNA strand integrity including breaking the connection of ester bond sequences and destroying base modifications. From the initial point of its life cycle, radiotherapy blocks various physiological functions of the tumor cell and genetic information delivery[40,41]. Cell cycle distribution has a deep influence on radiotherapy[40,42-45]. Flow cytometry analysis revealed that COS treatment leaves a large number of SW480 cells stranded in the G2/M phase that is very sensitive to radiation, while reducing the G1 phase and S phase that are responsible for DNA damage repair, reducing the resistance of cancer cells to radiotherapy and enhancing its therapeutic effect. Radiation biological research pointed out that in order to ensure a smooth and orderly replication, the whole proliferation process speed is controlled at G1, S and G2 levels, respectively, by three regulatory processes[46-49], speculating that COS control in cell cycle distribution is most likely related to the start-up and expression of these three processes.

In summary, COS not only directly arrests SW480 cell growth, but also helps radiotherapy. COS induces SW480 cells apoptosis accompanied by a large number of proliferation process changes. Thus, this greatly enhances radiation lethality and has a beneficial therapeutic effect. In-depth exploration of COS as radiosensitizer is expected to bring a new dawn for the life of colorectal cancer patients.

COMMENTS

Background

Modern unhealthy diets and living habits are major causes that stimulate the prevalence of colorectal cancer to continue to soar. In China, colorectal cancer has a prevalence of 16.14%, which has clinically already leapt up to fifth place among cancers; and the incidence in males is as high as 18.75%. In 2012, over 250000 cases of patients were added nationwide; this total accounts for 18.6% in the world. China has become a big focus in the forefront of colorectal cancer research, and efficient and low toxicity treatments are needed. Although radiotherapy has great significance for cancer treatment, killing cancer cells could injure healthy tissues, causing malignant complications. This has been a researcher's hurdle that is difficult to bypass. Radiosensitizers have now emerged as a timely aid.

Research frontiers

In order to reduce the toxicity of radiotherapy, current research has focused on primarily two aspects: increase in the accuracy of positioning, and enhancement of radiation radiosensitivity of tumor tissues. Using radiation sensitizers has become popular because it is simple to apply. Previous studies have shown that radiosensitization mechanisms include improved cell hypoxia, increased DNA damage and influence of the cycle phase distribution. In addition to 5-fluorouracil, cisplatin and gemcitabine, the conventional radiotherapy sensitizers, C225, L-778-123 and COX-2 inhibitors and other new sensitizers have gained attention in recent years. Further interdisciplinary approaches have also started to introduce new drugs and new mechanisms of action in the field of radiation sensitizer agents.

Innovations and breakthroughs

Derived from the depolymerization of chitosan, COS has been considered as the human healthy "almighty" guardian by the biomedical field. It can improve body acid-base balance, activate immune function, remove blood lipids, lower blood sugar, and regulate a variety of physiological activity. Particularly, it has anti-tumor effects, which has been a research focus by domestic and foreign scholars in recent years. Based on historical reports, COS has a widespread growth blocking effect on HL-60, RBL-2H3, SGC-7901 and tumor cell lines of other organs, and there are a variety of ways to achieve this effect. Although there are many reports about COS anticancer effects, there are few studies on the applications of radiation sensitization. In this study, human colon cancer SW480 cells were selected and treated with COS application in parallel with radiation, to verify that COS can enhance radiosensitivity in colorectal cancer cell lines.

Applications

This study showed that chitooligosaccharides can effectively enhance the sensitivity of SW480 cancer cells to radiation; chitosan oligosaccharide combined with radiotherapy treatment would be helpful and promising for colorectal cancer. Regarding the effect of chitooligosaccharides on radiosensitization, an in-depth study would be expected to explore this efficient and low-damage anticancer therapeutic breakthrough.

Peer-review

This is a very interesting study about the chitooligosaccharide promotion of radiosensitivity in a colon cancer line. The study is well designed and the manuscript is well written.

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ORIGINAL ARTICLE

Basic Study

Faecalibacterium prausnitzii supernatant ameliorates dextran sulfate sodium induced colitis by regulating Th17 cell differentiation

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Abstract

AIM: To explore the preventive and therapeutic effects of *Faecalibacterium prausnitzii* (*F. prausnitzii*) supernatant on dextran sulfate sodium (DSS) induced colitis in mice.

METHODS: Forty C57BL/6J male mice were randomly

divided into four groups: control group, model group, treatment group, and prevention group. Mice were weighed daily. On day 10, the colon length was measured, the colorectal histopathologic damage score (HDS) was assessed, and plasma interleukin (IL)-17A, IL-6, and IL-4 levels were detected by enzyme-linked immunosorbent assay. The expression of transcription factor retinoic acid-related orphan receptor-γt (RORγt) and IL-17A in colon inflammatory mucosa tissue were determined by immunohistochemical assay, and the expression levels of RORγt mRNA, IL-17A mRNA, and IL-6 mRNA were detected by real-time quantitative polymerase chain reaction (PCR). The proportion of Th17 in mononuclear cells in spleen was assayed by fluorescence activated cell sorter.

RESULTS: When compared with the model group, the colon length ($P < 0.05$) and body weight ($P < 0.01$) in the treatment and prevention groups were significantly increased, and the colon HDS was decreased ($P < 0.05$ and $P < 0.01$). There was no statistical difference between the treatment group and prevention group. After treatment with *F. prausnitzii* supernatant, the plasma levels of IL-17A and IL-6 ($P < 0.05$), the protein and mRNA expression of IL-17A and RORγt, and the Th17 cell ratio of spleen cells ($P < 0.01$) were significantly decreased compared to the model group. Plasma IL-4 level in the prevention group was significantly higher than that in the model group ($P < 0.05$), but there was no significant difference between these two groups in the expression of IL-6 in both the plasma and colon mucosa tissues.

CONCLUSION: *F. prausnitzii* supernatant exerts protective and therapeutic effects on DSS-induced colitis in mice, probably *via* inhibition of Th17 differentiation and IL-17A secretion in the plasma and colon mucosa tissues. It can also improve colitis in mice by downregulating IL-6 and prevent colitis by upregulating IL-4.

Key words: *Faecalibacterium prausnitzii*; Ulcerative colitis; Animal model; Th17 cell; Treatment; Prevention

Core tip: *Faecalibacterium prausnitzii* (*F. prausnitzii*) supernatant has anti-inflammatory and immune regulatory activity. This study showed that the preventive and therapeutic use of *F. prausnitzii* supernatant could ameliorate dextran sulfate sodium (DSS)-induced colitis in mice by inhibiting Th17 cell differentiation and inflammatory cytokines release.

Huang XL, Zhang X, Fei XY, Chen ZG, Hao YP, Zhang S, Zhang MM, Yu YQ, Yu CG. *Faecalibacterium prausnitzii* supernatant ameliorates dextran sulfate sodium induced colitis by regulating Th17 cell differentiation. *World J Gastroenterol* 2016; 22(22): 5201-5210 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i22/5201.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i22.5201

INTRODUCTION

Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), are multifactorial ailments characterized by intestinal inflammation. Although the precise etiology and pathogenesis of IBD are not fully elucidated, multiple factors contribute to IBD, including genetic background, environment, intestinal flora imbalance, and immune disorder[1-4]. It has been hypothesized that an undesired intestinal mucosal immune response to intestinal flora imbalance contributes to the onset of IBD in genetically susceptible individuals.

A T helper (Th)17 cell is defined as a cell producing the cytokine interleukin (IL)-17A, but it also can secrete many other cytokines, such as IL-17F, IL-6, and IL-23, during an inflammatory response[5]. Th17 cells are characterized by the expression of the transcription factor retinoic acid-related orphan receptor (RORγt), and there is growing evidence that Th17 cells are paramount in the development of human autoimmune diseases, including IBD[6-8]. In the intestine of IBD patients, elevated numbers of Th17 cells and increased RORγt and IL-17 levels are found[9]. The differentiation of Th17 cells from naïve CD4+ T cells is known to be affected by multiple cytokines, such as transforming growth factor (TGF)-β, IL-6, IL-4, and IL-23[10,11]. IL-6 plays a key role in cooperating with TGF-β to initiate Th17 differentiation, while IL-4 inhibits Th17 differentiation.

Faecalibacterium prausnitzii (*F. prausnitzii*) is the major bacterium of the Clostridium leptum group, and is one of the most abundant anaerobic bacteria in the human gut[12]. *F. prausnitzii* plays an important role in maintaining the intestinal health and providing energy to the colonocytes[13]. A recent study indicated that *F. prausnitzii* levels were decreased in IBD patients compared with healthy controls[14]. Previously, we confirmed in animals that both the bacteria and its supernatant relieved trinitro-benzene-sulfonic acid induced colitis in rats[15]. Nevertheless, the specific mechanism is largely unclear.

Dextran sulfate sodium (DSS) induced colitis is a well-established animal model for IBD pathogenesis, and it has been used in preclinical studies for over two decades[16,17]. Furthermore, it has been shown that the clinical features and pathological changes of DSS-induced colitis in mice were similar to human UC[18]. Here, we determined whether the *F. prausnitzii* supernatant could relieve DSS-induced colitis in mice by reducing Th17 cells and inflammatory cytokines.

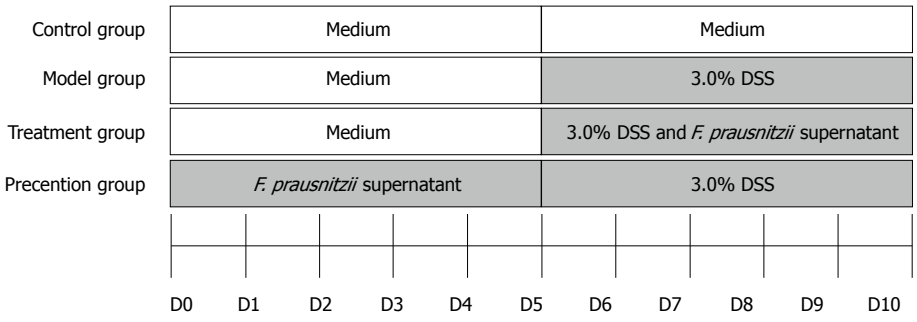


Figure 1 Flow diagram of the study design. DSS: dextran sulfate sodium; *F. prausnitzii*: *Faecalibacterium prausnitzii*.

MATERIALS AND METHODS

Animals

All experiments were approved by the Experimental Animal Ethical Committee of Nanjing Drum Tower Hospital, the Affiliated Hospital of Nanjing University Medical School. Forty male C57BL/6J mice aged 8-10 wk and weighing 18-22 g were obtained from the Animal Center, Nanjing Drum Tower Hospital (Nanjing, China). The mice were allocated equally and randomly to four groups: control group, model group, treatment group, and prevention group. The group divisible design is shown in Figure 1. The period of observation was 10 d. In the first 5 d, the mice in the prevention group were given supernatant of *F. prausnitzii* (five times concentrated, 0.1 mL/10 g) through gavage once a day, while the other groups received the same dosage of medium. For the next 5 d, all groups, except for the control group, were treated with 3.0% DSS in their drinking water *ad libitum*, the treatment group was fed *F. prausnitzii* supernatant by gavage once a day.

Mice were weighed daily and sacrificed by cervical dislocation at day 10. Colons were dissected, and the distance from cecum to anus was measured. The colon tissues were fixed in 4% formalin for later pathological examination and immunohistochemical study. The peripheral blood and spleen were isolated for testing Th17 cells and cytokines.

F. prausnitzii culture

F. prausnitzii (ATCC 27766, Manassas, VA, United States) was cultured anaerobically at 37 ℃ in LYHBHI medium [main component of brain-heart infusion medium (37 g/L, BD, Franklin Lakes, NJ, United States), yeast extract (5 g/L, Oxoid, Basingstoke, United Kingdom), cellobiose (1 g/L, Sigma, St. Louis, MO, United States), maltose (1 g/L, Amresco, Solon, OH, United States), hemin (5 mg/L, Sigma), and cysteine (0.5 g/L, Sigma)]. The number of live bacteria (colony-forming units, CFU) was calculated according to optical density (OD) at 600 nm. The supernatant was collected from cultures with 109-1010 CFU/mL (OD = 1.9). Sterile culture medium acted as placebo. Bacterial supernatant and sterile culture medium were lyophilized and stored at -80 ℃. They were thawed and diluted to five times concentrated solution with phosphate buffered saline (PBS) before administration.

Colon histopathologic grading

The histopathologic grading of colon damage was scored by two blinded pathologists under microscope based on Neurath Scoring criteria as previously described[19]. In short, 4: transmural leukocyte infiltrations, high vascular density, loss of goblet cells, and thickening of the colon wall; 3: high level of leukocyte infiltration, thickening of the colon wall, high vascular density; 2: low level of leukocyte infiltration; 1: very low level of leukocyte infiltration; and 0: no inflammation.

Isolation of splenic mononuclear cells

Splenic mononuclear cells were isolated from spleens through Ficoll–Isopaue density gradient centrifugation[20]. Fresh spleens were placed in Roswell Park Memorial Institute (RPMI)-1640 (Gibco, Carlsbad, NY, United States) and mechanically disrupted by a 2 mL syringe plunger into cell suspensions. Cell suspensions were repeatedly aspirated with a sterile Pasteur pipette and gently filtered through a 200 μm strainer. Splenic single-cell suspensions were layered over an equal volume of Ficoll-Hypaque Solution (Haoyang BioScience Corporation, Tianjin, China) per spleen and centrifuged at 1500 rpm for 20 min. The band of leukocyte enriched fraction at the interface was collected after centrifugation at 1800 rpm for 10 min without brake. The resulting splenic mononuclear cell density was counted in a hemocytometer, and viability was assessed by Trypan blue staining.

Fluorescence activated cell sorter analysis of Th17 in mononuclear cells

Flow cytometry followed routine procedures by using 2 × 10^6 cells per sample. The splenic mononuclear cells were stimulated by phorbol-12-myristate-13-acetate (PMA), ionomycin, and brefeldin A for 5 h at 37 ℃ in a 5% CO2 incubator, then labeled with fluorescein isothiocyanate (FITC) anti-mouse CD4 (eBioscience, San Diego, CA, United States) and APC anti-mouse CD3 (eBioscience). After permeabilization and fixed

Table 1 Polymerase chain reaction primers gene sequences

Target gene	Primer sequence	Product length (bp)
ROR-γt	forward: GACGGCCAACTTACTCTTGG reverse: AGAAACTGGGAATGCAGTGG	109
IL-17A	forward: TCCCTCTGTGATCTGGGAAG reverse: CTCGACCCTGAAAGTGAAGG	154
IL-6	forward: CGGAGAGGAGACTTCACAGAG reverse: CATTTCCACGATTTCCCAGA	105
GAPDH	forward: CATGGCCTTCCGTGTTCCTA reverse: TGTCATCATACTTGGCAGGTTTCT	83

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; IL: Interleukin; ROR-γt: Related orphan receptor-γt.

treatment, cells were labeled with PE anti-mouse IL-17 (eBioscience). The stained cells were tested by flow cytometry (BD, San Jose, CA, United States) and analyzed by the Cell Quest software.

Enzyme-linked immunosorbent assay cytokines in murine plasma

Cytokines (IL-17A, IL-6, IL-4) were measured using a commercially available enzyme-linked immunosorbent assay kit (Yunhan Biological Technology Corporation, Shanghai, China) according to the manufacturers' instructions.

Real-time quantitative polymerase chain reaction

Total RNAs were extracted from mid-colon samples taken from mice in each group using the Trizol reagent (Invitrogen, Carlsbad, CA, United States) with the following procedure. The concentration was determined by NanoDrop TM 1100 (NanoDrop Technologies, Wilmington, DE, United States). Total RNA was reversely transcribed into cDNA using reverse transcription kit. The polymerase chain reaction (PCR) reactions were performed in a 96-well Optical Reaction Plate (Applied Biosystems, Foster City, CA, United States) with the following procedure: degeneration 95 ℃ for 30 s, annealing 95 ℃ for 5 s, 40 cycles of 60 ℃ for 34 s. All primers and probes used in this study are listed in Table 1.

Immunohistochemistry

Paraffin slides of colon were re-hydrated in different concentrations of ethanol and washed in PBS. Sections were microwaved in sodium citrate buffer. After blocking with 10% goat serum for 30 min, sections were incubated with rabbit anti-rat IL-17 antibodies (Abcam, Cambridge, United Kingdom) overnight at 4 ℃. Slides were then incubated with the corresponding secondary antibody (Zsbio, Beijing, China), labelled with horseradish peroxidase, developed using a diaminobenzidine (DAB) reaction, and counterstained with hematoxylin. Cells stained with the antibodies were calculated by random selection of five fields under a microscope at 200 × magnification.

Statistical analysis

The GraphPad Prism version 5.0 (La Jolla, CA, United States) was used for data analysis. Data are presented as mean ± SD and were analyzed using one-way analysis of variance. $P < 0.05$ was considered to be statistically significant.

RESULTS

Symptoms and body weight of mice

Mice became symptomatic (*e.g.*, bloody diarrhea, weight loss, shakes andsloth) by day 3 of drinking 3.0% DSS *ad libitum*. The symptoms worsened with prolonged 3.0% DSS drinking time.

The mice in the model group had obvious weight loss compared to the control group ($P < 0.001$), and the mice from the model group weighed significantly less than those from the treatment and prevention groups. There was no significant difference in weight loss between the treatment group and prevention group (Figure 2).

Colon length and pathological changes

Compared with the control group, the mice in the model group had markedly shorter colon length (7.89 ± 1.536 *vs* 4.92 ± 0.925, $P < 0.001$), more serious colon damage, and higher histopathologic damage scores (0.8 ± 0.632 *vs* 3.7 ± 0.483, $P < 0.01$). Histological examination of model group mice showed that the normal colon mucous membrane structure disappeared, extensive ulceration developed, and a large number of inflammation cells infiltrated. However, culturing supernatant of *F. prausnitzii* in treatment and prevention group mice significantly ameliorated the colon damage by increasing colon length ($P < 0.01$ and $P < 0.05$) and reducing high histopathologic damage scores ($P < 0.05$) as compared with model group (Figure 2).

Th17 cell percentage change in splenic mononuclear cells

The ratio of Th17 cells in splenic mononuclear cells of the model group was significantly higher than that of the control group (4.02 ± 1.111 *vs* 1.34 ± 0.417, $P < 0.001$). It was obviously decreased after preventive and therapeutic application of *F. prausnitzii* supernatant (4.02 ± 1.111 *vs* 2.60 ± 0.839, $P < 0.01$ and 4.02 ± 1.111 *vs* 2.21 ± 1.030, $P < 0.05$), and there was no significant difference between the treatment and prevention groups (Figure 3).

IL-17A, IL–6, and IL-4 levels in peripheral plasma

Plasma IL-17A, IL-6, and IL-4 levels of the control group were significantly different from the model group [15.73 ± 4.382 (pg/mL) *vs* 28.44 ± 4.116 (pg/mL) $P < 0.01$, 81.19 ± 13.609 (pg/mL) *vs* 111.82 ±

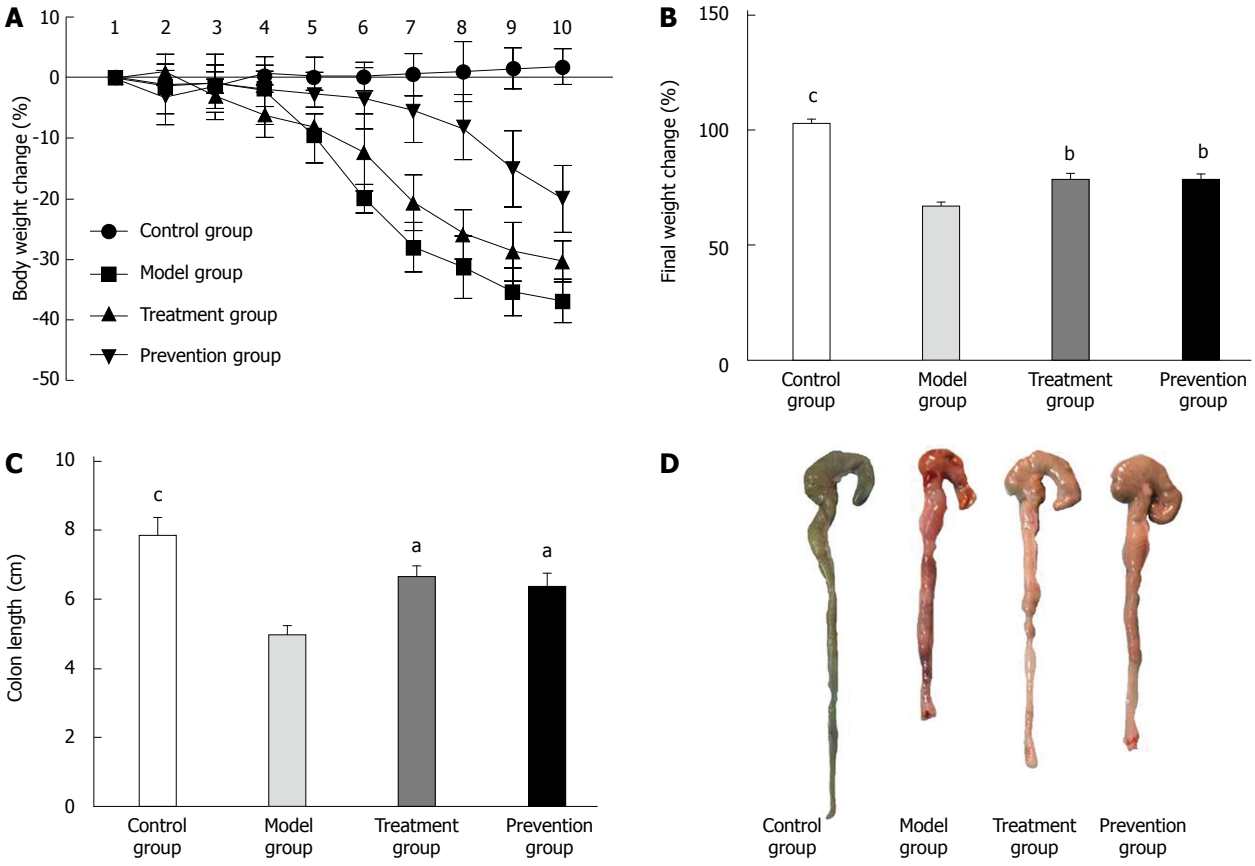


Figure 2 Body weight and colonic length in mice. A, B: Body weight change; C, D: Colon length. Data are the mean ± SD. n = 8-10. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* model group.

14.369 (pg/mL) $P < 0.05$, 79.91 ± 12.245 (pg/mL) *vs* 38.16 ± 9.507 (pg/mL) $P < 0.001$]. The plasma levels of IL-17A in the treatment and prevention groups were significantly lower than that in the model group ($P < 0.05$). Plasma IL-6 level in the treatment group was also significantly less than that in the model group ($P < 0.05$), but the difference was not statistically significant between the prevention group and model group. On the contrary, level of plasma IL-4 in the prevention group was obviously higher than that in the model group ($P < 0.05$), while no difference was found between the treatment group and the model group (Figure 3).

Expression of cytokines and RORγt mRNA in colon mucosal tissue

The expression of IL-17A, IL-6, and RORγt mRNA in colon tissue of mice in the model group was significantly higher than that in the control ($P < 0.001$) and treatment groups ($P < 0.05$). When compared with the model group, the expression of IL-17A and RORγt mRNA in colon inflammatory tissue of the treatment and prevention groups was significantly decreased ($P < 0.01$ or $P < 0.05$). There was no difference, however, in IL-6 between the model group and prevention group. As shown in Figure 4, the expression of cytokines and RORγt mRNA in colon mucosal tissue did not significantly differ between the treatment and prevention groups.

Immunohistochemistry

To investigate the effects of IL-17A and RORγt on colon tissue, we conducted immunohistochemical staining of proinflammatory cytokines in tissue sections. Consistent with the results of quantitative real time PCR, the expression of IL-17A and RORγt in the colon tissue of model group mice was significantly increased compared to that in the control group ($P < 0.001$) and treatment group ($P < 0.05$). Although the expression of RORγt in colon tissue was declined after protective use of *F. prausnitzii*, there was no difference between the model and prevention groups (Figure 5).

DISCUSSION

In this study, we found that *F. prausnitzii* supernatant ameliorated colitis in mice by regulating Th17 cell differentiation and inhibiting the excretion of relevant inflammatory cytokines. We also found that *F. prausnitzii* supernatant was effective in the treatment and prevention of DSS-induced mice colitis by inhibiting differentiation of Th17 cell.

Both living *F. prausnitzii* and *F. prausnitzii* supernatant, which contains a mixture of secreted products,

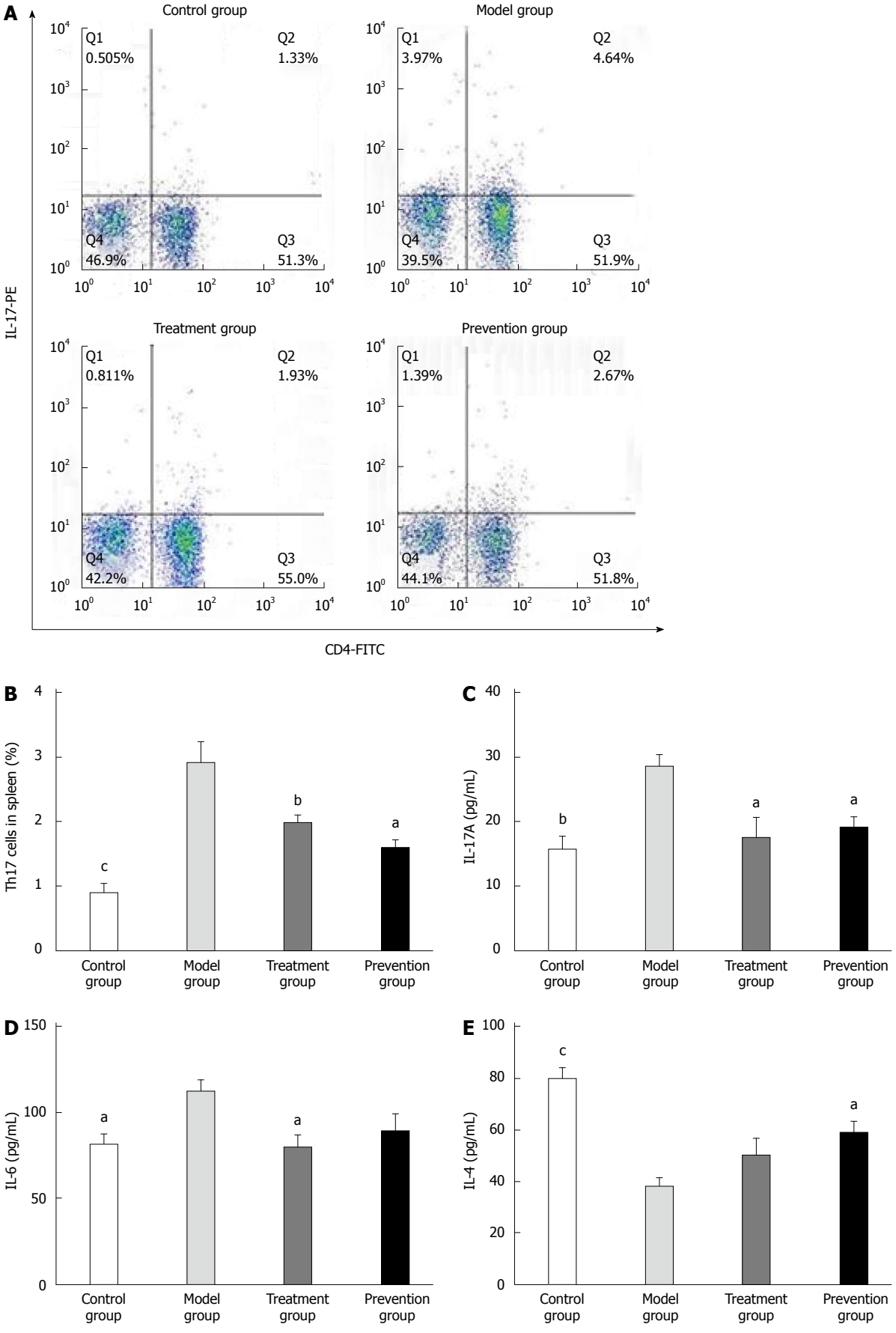


Figure 3 Proportion of Th17 cells in splenic mononuclear cells and plasma cytokines levels. Flow cytometry figures (A) and statistical analysis (B) of Th17 cell in each group of the mice splenic MNC. Plasma IL-17 A (C), IL–6 (D) and IL-4 (E) levels by enzyme-linked immunosorbent assay. Data are the mean ± SD. n = 8-10. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* model group. IL: Interleukin; MNC: mononuclear cells.

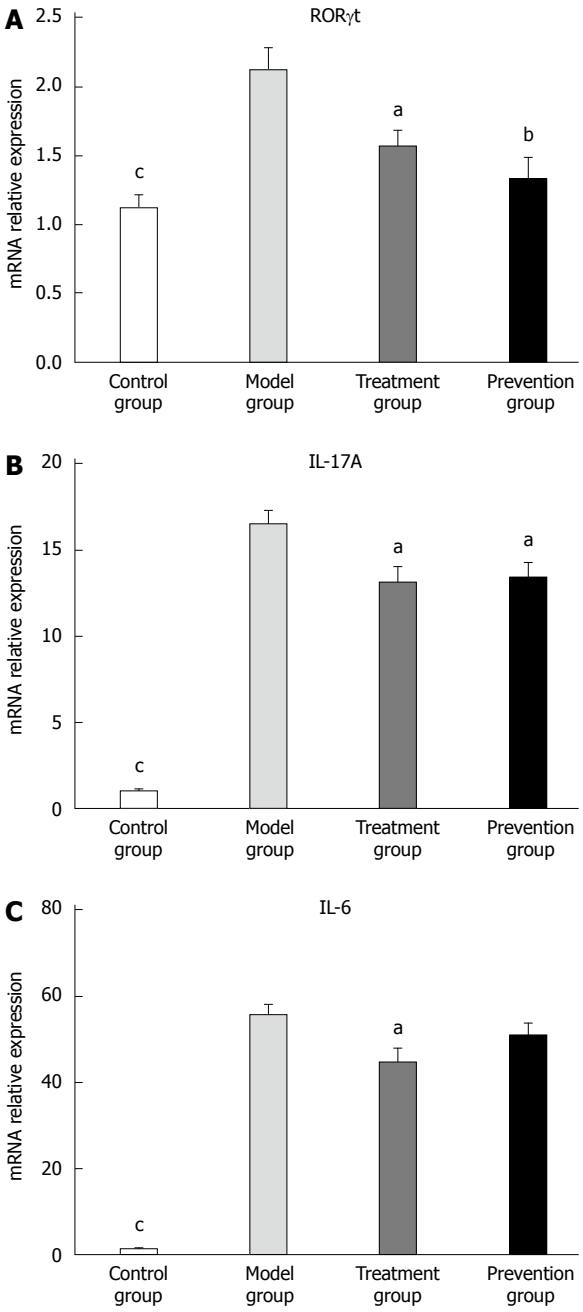


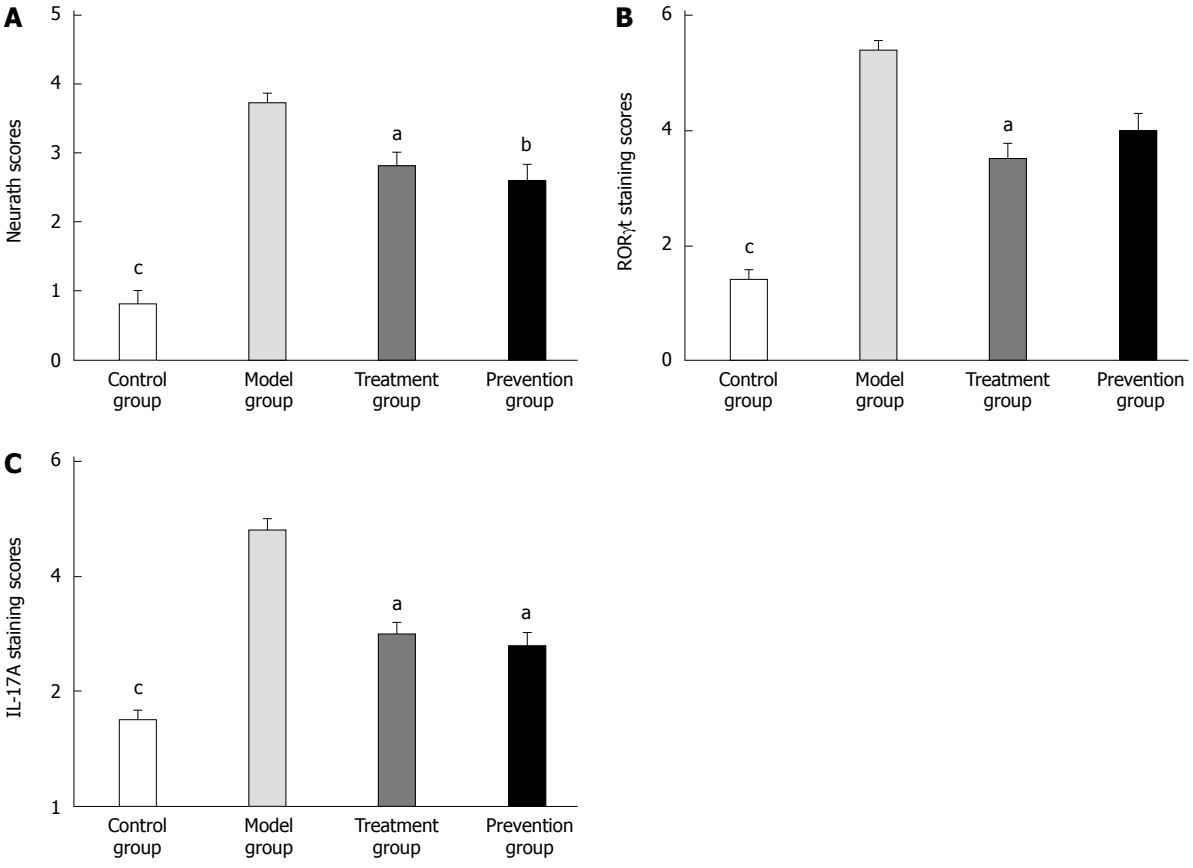
Figure 4 Cytokine mRNA expression in colon mucosal tissue. A: RORγt mRNA; B: IL-17A mRNA; C: IL-6 mRNA. Data are the mean ± SD. n = 8-10. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* model group. ROR-γt: Related orphan receptor-γt; IL: Interleukin.

have been shown to have an anti-inflammatory effect[21]. Compared to *F. prausnitzii*, its supernatant could be more effective therapeutically, as it may have a longer shelf-life, which would facilitate delivery, handling, and administration[22]. However, the exact composition and the anti-inflammatory mechanism of *F. prausnitzii* supernatant are currently largely unknown. Therefore, we explored the effects and immune mechanisms of *F. prausnitzii* supernatant on DSS-deduced colitis. Our study showed that the plasma levels of IL-17A and IL-6, the protein and mRNA expression of IL-17A and RORγt in intestinal mucosa, and the Th17 cell ratio of spleen cells ($P < 0.01$) in supernatant treatment group were significantly decreased compared to those in the model group. This finding indicated that the therapeutic use of *F. prausnitzii* supernatant could ameliorate DSS-induced colitis through inhibiting Th17 cells. Carlsson *et al*[23] previously demonstrated that the supernatant of *F. prausnitzii* affected the function of the intestinal barrier.

Th17-related gene polymorphisms are associated with IBD susceptibility[24]. Th17-derived cytokines, such as IL-17A, IL-6, and IL-22, have been shown to be upregulated in the inflamed intestine of IBD patients[25,26]. IL-17A is a strong inflammatory cytokine, which can enhance cell permeability and promote the generation of other pro-inflammatory cytokines and chemokines[27]. Animal experiments, however, have found that neither IL-17A knockout nor neutralization of IL-17 could protect DSS-administrated mice from colitis, suggesting that the role of IL-17 in intestinal inflammation may not be entirely pathogenic[14,28]. Adequate expression of IL-17A plays an important role in maintaining intestinal immune function. Consistent with previous studies, we found that IL-17A levels in the plasma, spleen, and colon tissue were significantly increased in mice with colitis and that these levels were remarkably downregulated in mice treated with *F. prausnitzii* culture supernatant. Therefore, *F. prausnitzii* supernatant could attenuate DSS-induced mice colitis, possibly by inhibiting the expression of IL-17A[15,29].

We also found that levels of IL-6 in plasma and colon tissues of colitis mice were significantly reduced after *F. prausnitzii* supernatant treatment. *F. prausnitzii* supernatant could alleviate mice colitis by downregulating IL-6 levels and inhibiting Th17 cell differentiation, thus leading to reduced secretion of inflammatory cytokines (such as IL-17A and IL-6) and attenuation of the local inflammatory response. However, the regulation of IL-6 expression in the treatment and prevention groups was inconsistent, suggesting that there might be other ways of inhibiting Th17 differentiation. Fu *et al*[29] demonstrated that boosting of Th2 associated cytokines (IL-4, IL-13, and IL-10) can reverse Th17-mediated intestinal inflammation. We also found that plasma IL-4 levels in mice of the prevention group were significantly greater than those in the model group.

In conclusion, *F. prausnitzii* supernatant can prevent DSS-deduced colitis in mice by inhibiting the generation of Th17 cells in the spleen and intestinal mucosa, leading to a reduction of IL-17A and IL-6 levels and attenuation of intestinal inflammation. This study provides the theoretical basis for the application of *F. prausnitzii* supernatant in UC treatment and prevention. However, what specific substances in the supernatant of *F. prausnitzii* possess biological activity needs to be elucidated in future studies. The safety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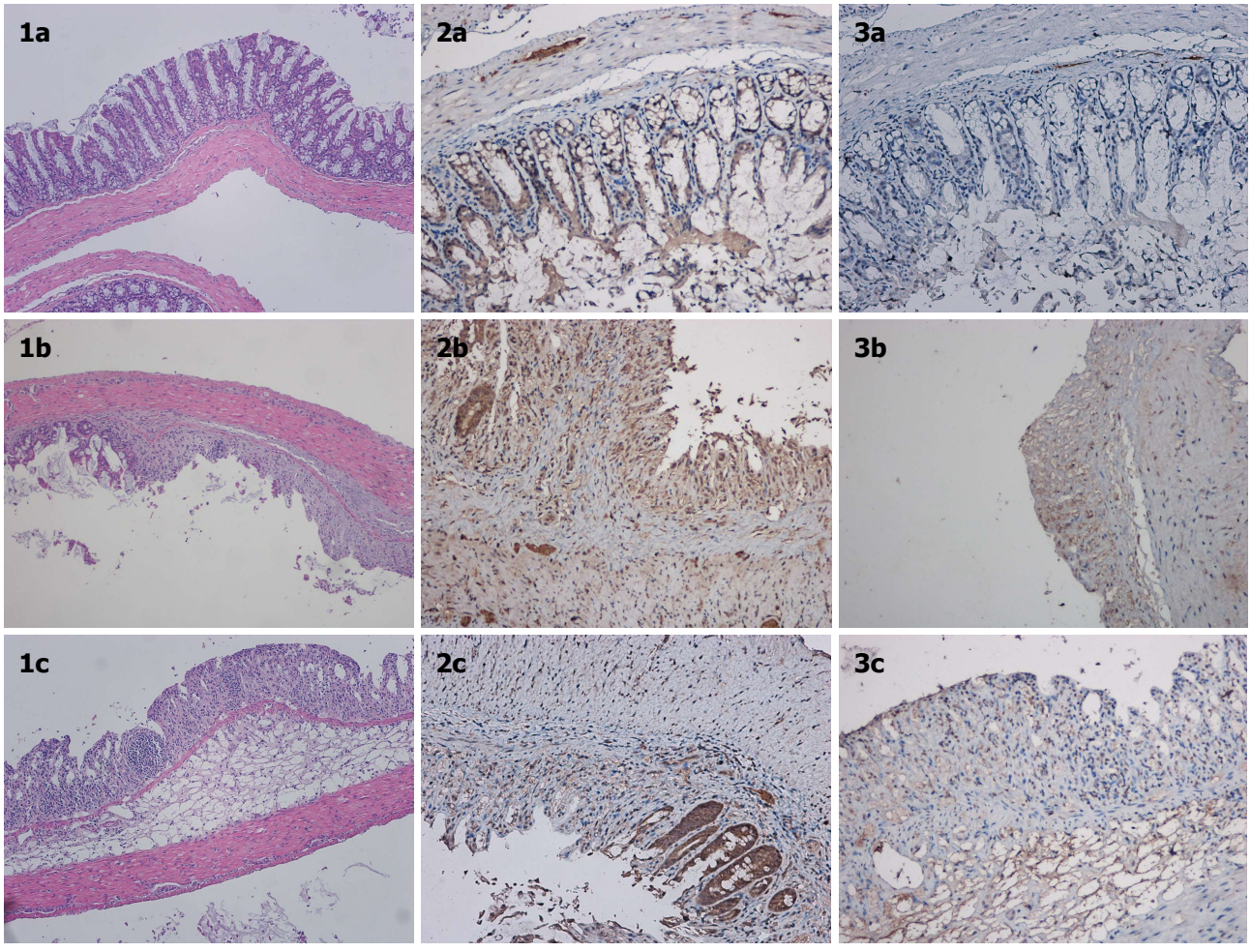
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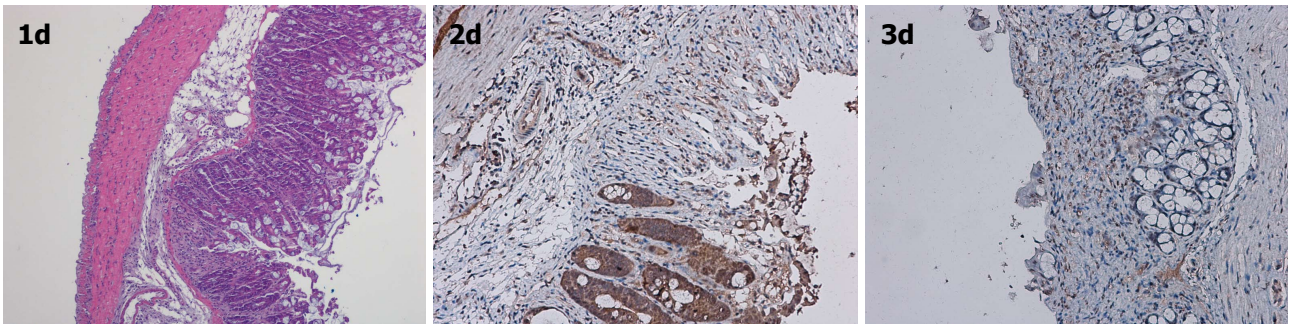

Figure 5 Colon Neurath Scores and related orphan receptor-γt and interleukin-17A protein expression. Colon Neurath Scores (A, 100 magnifications), RORγt (B, 200 magnifications), and IL-17A (C, 200 magnifications) protein expression in mice colon. Representative images of mice colonic mucosa (1a-1d). Representative immunohistochemical staining of RORγt (2a-2d) and IL-17A (3a-3d) in mice colon mucosa. Control group (a); model group (b); treatment group (c); prevention group (d). Data are the mean ± SD. n = 8-10. $^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.001$ *vs* model group.

and efficacy of *F. prausnitzii* supernatant also warrant further investigation by more large scale clinical trials.

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COMMENTS

Background

Inflammatory bowel disease (IBD) is a multifactorial ailment characterized by intestinal inflammation, and its etiology is complicated and ambiguous. Factors that contribute to IBD include genetic background, environment, intestinal flora imbalance, and immune disorder as well as the interactions between them.

Research frontiers

Faecalibacterium prausnitzii (*F. prausnitzii*) is a common anaerobic bacteria that colonizes the human gut, and it plays a critical role in IBD. *F. prausnitzii* supernatant has anti-inflammatory and immune regulatory activity. Previously, the authors showed in animals that both the bacteria and its supernatant relieved trinitro-benzene-sulfonic acid-induced colitis in rats. However, the specific mechanism is largely unclear.

Innovations and breakthroughs

This study is the first to show that the preventive and therapeutic use of *F. prausnitzii* supernatant could ameliorate dextran sulfate sodium (DSS) induced mice colitis through inhibiting Th17 cells. The molecular mechanism of proliferation and differentiation of Th17 cells was different. *F. prausnitzii* supernatant may treat colitis in mice by downregulating IL-6 and preventing the upregulation of IL-4.

Applications

This study investigated the molecular mechanism of the preventive and therapeutic use of *F. prausnitzii* supernatant for IBD and provided evidence for the prevention and treatment of the disease.

Terminology

F. prausnitzii is the major bacterium of the Clostridium leptum group and is one of the most abundant anaerobic bacteria in human gut.

Peer-review

The study investigates the preventive and therapeutic role of *F. prausnitzii* supernatant in a mouse model of DSS-induced ulcerative colitis. The topic is interesting, and the design and methods have clear scientific values. The data are clear and well presented.

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ORIGINAL ARTICLE

Basic Study

Visceral hypersensitive rats share common dysbiosis features with irritable bowel syndrome patients

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Data sharing statement: Data will be available for scientific sharing. The sra number for the sequencing data is pending.

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Abstract

AIM: To evaluate gut microbial dysbiosis in two visceral hypersensitive models in comparison with irritable bowel syndrome (IBS) patients and to explore the extent to which these models capture the dysbiosis of IBS patients.

METHODS: Visceral hypersensitivity was developed using the maternal separation (MS) rat model and post-inflammatory rat model. The visceral sensitivity of the model groups and control group was evaluated using the abdominal withdraw reflex score and electromyography in response to graded colorectal distention. The 16S ribosomal RNA gene from fecal samples was pyrosequenced and analyzed. The correlation between dysbiosis in the microbiota and visceral hypersensitivity was calculated. Positive findings were compared to sequencing data from a published human IBS cohort.

RESULTS: Dysbiosis triggered by neonatal maternal separation was lasting but not static. Both MS and post-inflammatory rat fecal microbiota deviated from that of

the control rats to an extent that was larger than the co-housing effect. Two short chain fatty acid producing genera, *Fusobacterium* and *Clostridium XI*, were shared by the human IBS cohort and by the maternal separation rats and post-inflammatory rats, respectively, to different extents. *Fusobacterium* was significantly increased in the MS group, and its abundance positively correlated with the degree of visceral hypersensitivity. *Porphyromonadaceae* was a protective biomarker for both the rat control group and healthy human controls.

CONCLUSION: The dysbiosis MS rat model and the post-inflammatory rat model captured some of the dysbiosis features of IBS patients. *Fusobacterium*, *Clostridium XI* and *Porphyromonadaceae* were identified as targets for future mechanistic research.

Key words: Animal model; Irritable bowel syndrome; Microbiota; Pyrosequencing; 16S rRNA gene

Core tip: Dysbiosis of the gastrointestinal microbiota and hypersensitivity to colonic distension are critical features of irritable bowel syndrome (IBS). For animal models, the correlation between dysbiosis in the microbiota and visceral hypersensitivity remains unknown. This study identified common biomarkers between the animal models and IBS patients, which may be targets for future mechanistic research.

Zhou XY, Li M, Li X, Long X, Zuo XL, Hou XH, Cong YZ, Li YQ. Visceral hypersensitive rats share common dysbiosis features with irritable bowel syndrome patients. *World J Gastroenterol* 2016; 22(22): 5211-5227 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i22/5211.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i22.5211

INTRODUCTION

The human intestinal tract is home to trillions of bacteria that have co-evolved with their host over millennia[1]. Their combined genomes, called a metagenome, contain 150-fold more genes than do the human hosts, and they provide functions that humans otherwise do not have[2]. Complex interactions exist between the gut microbiota and the host[3]. Irritable bowel syndrome (IBS) is a common gastrointestinal disorder that is characterized by abdominal pain and alterations in bowel habits; statistically, IBS affects 7%-10% of people worldwide[4]. Accumulating evidence has indicated that the gut microbiota may participate in the pathogenesis of IBS[5]. Because collecting fecal samples both before and after a gastrointestinal infection from the same IBS patients is unfeasible for clinics, only gut dysbiosis in standing IBS patients has been evaluated to date[6,7]. However, how gut microbiota abnormalities arise and are maintained over time is unclear. These questions are critical for interventions targeting the microbiota, such as probiotic usage. In this work, we used visceral hypersensitive rat models to investigate the longitudinal changes of gut microbiota.

Currently, both post-infectious/inflammatory models and stress-related models have been frequently used to study the pathophysiology of IBS[8,9]. There are more than 12 major post-infectious/post-inflammatory models to mimic post-infectious IBS, which occurs after an initial episode of acute gastrointestinal infection. Chemicals such as trinitrobenzene sulfonic acid (TNBS)[10], mustard oil[11] and dextran sulfate sodium[12] were used to cause mucosal injury in the post-inflammatory models, and pathogens such as *Trichinella spiralis*[13] and *Campylobacter*[14] were used to infect the gut; both led to visceral hypersensitivity. Stress-related models[15] could also induce the modulation of visceral pain, and this may involve changes in the brain-gut axis[9]. However, one of the unsolved problems is the extent to which these models recapture the characteristics of gut dysbiosis in IBS patients. In this work, we used two visceral hypersensitive models, the TNBS post-inflammatory (pTNBS) model and the maternal separation (MS) model, to investigate: (1) whether and the extent to which these models reproduce the disturbance of gut microbiota in a similar way to that of the IBS patients; and (2) whether microbial dysbiosis, if it exists, is static or shifting in these visceral hypersensitive models. We also hoped to identify targets in the models' gut microbial communities that are suitable for use in developing probiotics to specifically modulate the microbiota.

MATERIALS AND METHODS

Animal maintenance and modeling

Sprague-Dawley rats were purchased from the animal center of Shandong University of Traditional Chinese Medicine. The rats were allowed to habituate for 7 d to the breeding facility prior to mating. They were kept under standardized specific pathogen-free conditions (21-22 ℃, 12:12-h light-dark cycle) with access to pellet food and water *ad libitum*. All experiments were approved by the Ethical Committee and Institutional Animal Care and Use Committee of Qilu Hospital (KYLL-2013-005), and the methods were performed in strict accordance with the Animal Management Rules of the Chinese Ministry of Health. The overall design and co-housing relationship of involved rats are indicated in Figure 1A.

The MS visceral hypersensitive models were developed as previously described[15]. Briefly, rat pups that were randomly assigned to the MS group were stressed by separating them from their mothers for 3 h daily between postnatal days 2-14. The control group (Ctl) received normal breeding during this session. All pups were weaned on postnatal day 22, and only the

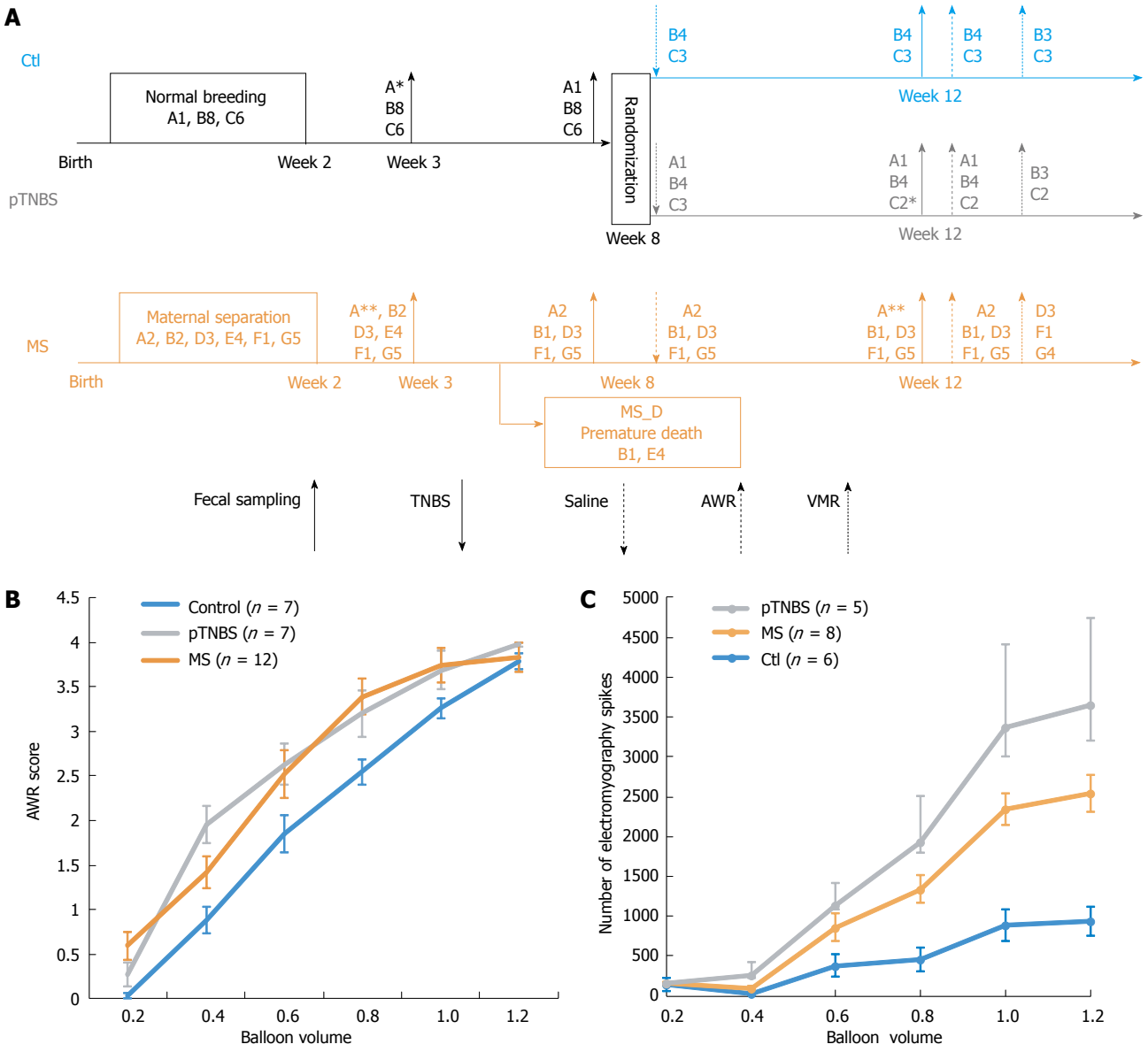


Figure 1 Study design and visceral sensitivity evaluation. A: Schematic flow chart showing the treatment and co-housing relationship of involved rats. For each code, such as "B8", the character "B" indicates the nest and number 8 indicates the number of rats. They were cohoused until "B4", which indicates that four of them were randomly chosen and cohoused together. The asterisk indicates fecal samples that failed to return the sequencing data; B: Abdominal withdrawal reaction (AWR) score in response to the graded colorectal distention (CRD); C: Visceromotor response (VMR) score in response to graded CRD. MS: Maternal separation; MS_D: MS early death; pTNBS: TNBS post-inflammatory.

male pups were used for the following study. Because some MS pups naturally died before they aged, 5 male rats were randomly chosen from those that were sampled at week 3 but did not survive to week 8. We indicated this group as the MS early death (MS_D) group. By including the MS_D group, we could test whether the dysbiosis caused by MS stress was more severe in the early dying pups.

After the second fecal collection at week 8, half of the control group was randomly assigned to the post-TNBS inflammation group (pTNBS). The pTNBS group was fasted for 24 h with free access to tap water, and then 0.4 mL of 5% (v/v) TNBS (P2297, Sigma, Shanghai, diluted to 0.8 mL using 50% ethanol) was administered into the colorectum.

Visceral hypersensitivity evaluation

After the last fecal collection at week 12, visceral hypersensitivity was evaluated using both the abdominal withdraw reflex (AWR) score and electromyography in response to graded colorectal distention (CRD). Graded CRD was induced by rapidly injecting (0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mL) saline into a urinary catheter balloon placed in the colon over 1 s and maintaining the distention for 20 s. AWR score was recorded according to a previously described method[16]. To represent the overall visceral sensitivity, a visceral hypersensitive index (VHI) for each rat was calculated by summing the rank of the AWR score at 0.4, 0.6, 0.8 and 1.0 of the total balloon volume.

The visceromotor responses (VMRs) to CRD were

quantified through electromyography of the rat obliqus external abdominis. Briefly, 5 d after embedding an electrode in the rat obliqus externus abdominis, the raw electromyography was recorded, rectified and quantified by counting the increased spike bursts during a 20 s window after graded CRD stimulation. The VMR index for each rat was calculated by summing the rank of electromyography spikes at 0.4, 0.6, 0.8 and 1.0 of the total balloon volume.

Fecal sample collection, DNA extraction, and pyrosequencing

Fecal samples were collected 3, 8 and 12 wk after birth. The samples from the pTNBS group at weeks 3 and 8 were indicated as Ctl-pTNBS and were analyzed as the controls because their treatment was the same as that for the Ctl group. A chart illustrating the overall treatment, fecal collection and model evaluation time points is shown in Figure 1A.

The samples were snap-frozen in liquid nitrogen and stored at -80℃. Genomic DNA was extracted with a TIANamp Stool DNA Kit according to the manufacturer's instructions (Cat# DP328, Tiangen, Beijing). DNA purity and concentration were measured using a Nanodrop2000 (Thermo Fisher). The DNA samples were shipped to Majorbio (Shanghai), where the DNA integrity check, PCR amplification, DNA quantification, emPCR (using Roche GS FLX Titanium emPCR Kits) and pyrosequencing of the 16S rRNA gene V3 to V1 region (using Roche Genome Sequencer FLX+) were performed according to their optimized protocols. The sequencing results were archived in the Short Reads Achieve (number pending).

Taxonomy quantification using 16S rRNA gene sequences

Raw sequencing data were prepared using Mothur v 1.33.0 according to their proposed 454 SOP (http://www.mothur.org/wiki/454_SOP)[17]. The raw sff files were decoded, denoised, trimmed and then aligned to Silva references (Release 102) using the default parameters. Chimeras were detected using the chimera.uchime command and were then removed. Distances between sequences were calculated with a cutoff value of 0.15. The sequences were clustered to the same operational taxonomic units (OTUs) if their distances were less than 0.03. The Shannon index and the inverse Simpson index (1/D) were calculated to indicate the diversity in each sample. Both indexes were calculated using Mothur, and the detailed formula can be accessed online (http://www.mothur.org/wiki/Shannon and http://www.mothur.org/wiki/Simpson). The OTU table was converted to biom files and the taxa abundance from domain to genus levels was generated using the summarize_taxa.py command in QIIME v1.8.0.

Statistical analysis

The richness of each taxonomy and the Shannon index between groups were compared using the Kruskal-Wallis test (KW) or a student's *t*-test in SAS V.9.3 statistical software. The heatmap plot with dendrograms was drawn using the heatplot function in the made4 packages in R (version 3.1.1). For primary component analysis (PCA), the axis value of all 80 samples was calculated together using the prcomp function in the stats package in R, and then the samples were plotted by each time point (week 3, 8, and 12). Within each time point, samples were clustered based on the Euclidian distance using the vegdist and hclust in the vegan package. According to the cluster results, the PCA plot points were grouped and connected using the ordispider and ordiellipse functions, where the ellipse was estimated to cover 75% of the dots in this group. The distribution of each group in each cluster was checked using Fisher's exact test in SAS. The community dissimilarity was tested by the weighted and unweighted UniFrac test using Mothur. The specific taxa that were differentially present in each group were identified using the LEfSe [linear discriminant analysis (LDA) coupled with effect size measurements] method with an LDA cut-off value of 2.0[18]. The Spearman correlation between the VHI and the taxonomy richness was calculated using the cor.test function in the stats package in R.

Comparing rat models and human IBS cohort

We downloaded the published 16S rRNA V4 region Miseq sequencing data by Jeffery *et al*[6]. This data set was analyzed by the same pipeline described above. We used LEfSe analysis on this dataset. Each positive finding from the rat experiment was checked against the human cohort. The relationship between human and rat biomarkers was indicated using a Venn plot.

RESULTS

Modeling and visceral hypersensitivity evaluation

The design and co-housing relationship of the rats involved in this study is shown in Figure 1A. Twenty-six of the 27 rats in this study (7 Ctl, 7 pTNBS, and 12 MS, see Figure 1B) were evaluated using AWR. The VHI score was calculated by summing the rank of the AWR score at 0.4, 0.6, 0.8 and 1.0 of the total balloon volume. A significant difference existed in the VHI among the three groups (χ^2 = 9.98, *df* = 2, *P* = 0.0068, KW). The VHI difference in the pTNBS to Ctl comparison was 38.4 (95%CI: 15.7 to 61.0, *P* < 0.05), and the VHI difference in the MS to Ctl comparison was 32.9 (95%CI: 12.7 to 53.0, *P* < 0.05). There was no significant difference in the MS to pTNBS comparison, with a VHI difference of 5.48 (95%CI: -14.7 to 25.7, *P* > 0.05). Nineteen of the 27 rats were evaluated by VMRs (Figure 1C). The VMR index for each rat was calculated by summing the rank of electromyography spikes at 0.4, 0.6, 0.8 and 1.0 of the total balloon volume. The VMR index among groups was insignificant although the control group tended to

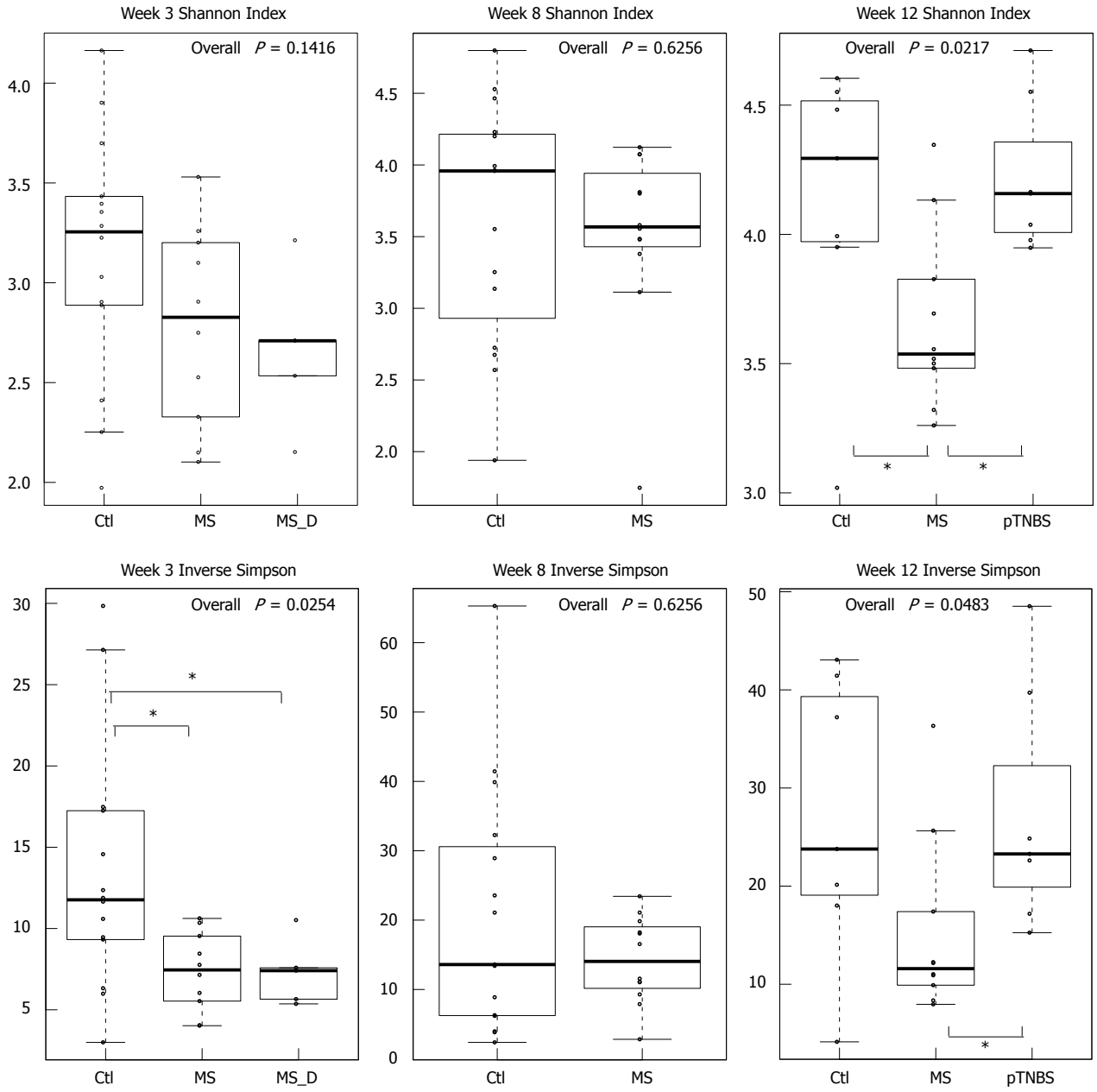


Figure 2 Microbial diversity. Shannon index (upper panel) and inverse Simpson (lower panel) of fecal microbiota at weeks 3, 8 and 12. Asterisk indicates $P < 0.05$ in pairwise comparison. MS: Maternal separation; MS_D: MS early death; pTNBS: TNBS post-inflammatory.

be lower than the MS and pTNBS groups (43.5 *vs* 86.5 and 60, respectively, $\chi^2 = 2.235$, $df = 2$, $P = 0.3271$, KW). Overall, these data indicate that both the MS and pTNBS groups developed visceral hypersensitivity at a comparable level.

DNA sequence data and microbial diversity comparison

A total of 489556 valid reads were assigned to 80 sequenced samples after barcode trimming. Sequence length varied between 230 and 327 bp per read. After removing chimeras and non-bacterial reads, 434594 reads remained. Each fecal sample included 3313 to 8161 reads. Based on a 97% species similarity, 2413 OTUs were identified from all of the fecal samples. Good's coverage for each sample varied from 95.57% to 99.72%. The rarefaction curve reached a plateau for most samples, suggesting that the present study captured the dominant phylotypes.

We first compared the microbial diversity among groups using the Shannon index and the inverse Simpson index (Figure 2). By week 3, the inverse Simpson index was significantly higher in the Ctl group ($\chi^2 = 7.34$, $df = 2$, $P = 0.0254$, KW). By week 8, the Shannon index and the inverse Simpson index were similar between the control group and the MS group. By week 12, the MS group has the lowest Shannon index ($\chi^2 = 7.67$, $df = 2$, $P = 0.217$, KW) and inverse Simpson index ($\chi^2 = 6.06$, $df = 2$, $P = 0.0483$, KW) compared with other groups. The pTNBS group had roughly same diversity indexes compared to the Ctl

group. These data indicate that the MS model, but not the pTNBS model, developed fecal microbiota with reduced diversity in a non-static manner.

Dysbiosis of major phyla

We then investigated whether differences in the phylum abundance exist at different time points (Figure 3). *Bacteroidetes* was the dominant phylum across all samples, and *Firmicutes* and *Proteobacteria* were the second and third most abundant phyla. No significance was reached for these three phyla at any of the 3 time points. The *Firmicutes* to *Bacteroidetes* (F/B) ratio was not significantly different ($P > 0.05$, KW).

Fusobacteria was abundant by week 3 (up to 0.25) and dropped to zero in most control rats. No difference in *Fusobacteria* existed by week 3 and week 8; however, by week 12, the MS group had significantly more *Fusobacteria* ($\chi^2 = 6.83$, $df = 2$, $P = 0.0328$, KW, $P < 0.05$ in MS-Ctl comparison). The control group had significantly more *Actinobacteria* than the MS group at week 8 ($P = 0.0034$, $\chi^2 = 8.58$, $df = 1$, KW). However, by week 12, the pTNBS group had significantly more *Actinobacteria* than the Ctl and MS groups ($\chi^2 = 8.07$, $df = 2$, $P = 0.0176$, KW, $P < 0.05$ in the pTNBS-Ctl and pTNBS-MS comparison). These data suggest that the dysbiosis of the major phyla may be phase dependent and different among the visceral hypersensitive rat models.

PCA and cluster analysis

We based the cluster analysis and PCA on the OTU data from the 16S rRNA gene pyrosequencing. The primary components for all 80 samples were calculated from the relative abundance of the 2413 OTUs. The relative importance of the first 20 primary components is plotted in Figure 4A. Primary component 1 and primary component 2 explained 27.7% and 14.6% of the total variance, respectively (Figure 4A). The differences in the primary components between the time points and experimental groups are listed in Table 1. Primary component 1 mainly reflected the effect of time points ($\chi^2 = 37.7$, $df = 2$, $P = 0.0000$, KW). Primary components 2 and 4 reflected the effect of experimental groups on fecal microbiota composition. The other 3rd, 6th, and 9th components were different both among time points and among groups.

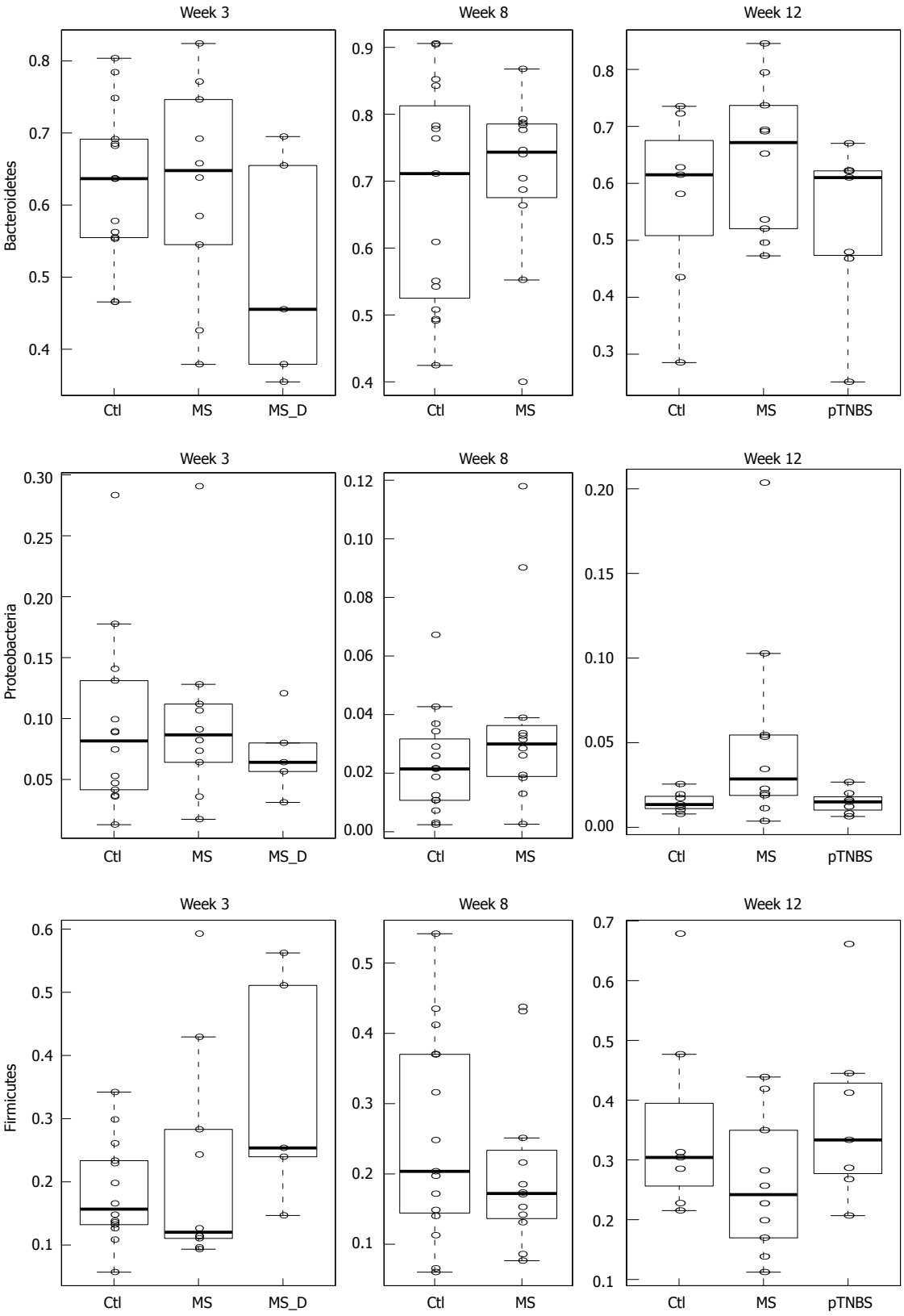
We then used cluster analysis to test whether the groups would fall into the same or different clusters. At each time point, the samples were fitted into the 3 top clusters based on the Euclidean distance. We designated the names of each cluster according to the samples it included. By week 3, the normal cluster included 8 Ctl, and the MS cluster included 4 MS and 4 MS_D samples. The mixed cluster included 6 Ctl, 6 MS and 1 MS_D samples (Figure 4B). The 3 groups' distribution in the clusters was significantly different ($P = 1.702 \times 10^{-4}$, Fisher's test). This result suggests that MS caused dysbiosis in rat models at early ages.

By week 8, the 27 fecal microbiota samples clustered into 3 mixed clusters (Figure 4C). The control group dominated mixed cluster 3 (12/16) while the MS group dominated mixed cluster 2 (7/8). The cluster distribution of the MS and control groups was significantly different ($P = 0.0114$, Fisher's test). By week 12, the 24 fecal microbiota samples formed 3 clusters (Figure 4D). The MS cluster included 5 MS samples, and the mixed cluster included roughly the same number of samples from the Ctl ($n = 6$), MS ($n = 5$), and pTNBS ($n = 7$) groups. Another "orphan" cluster included only one control sample. The difference among groups was significant ($P = 0.0150$, Fisher's test). These data suggest that the dysbiosis triggered by MS during childhood is still substantial in a fraction of adult rats. Four weeks after TNBS administration, the fecal microbiota of the post-inflammatory rat model was more similar to that of the control group as revealed by PCA and cluster analysis.

We tracked the longitudinal dysbiosis of 23 rats whose fecal samples were collected at all 3 time points. We analyzed whether the 10 MS rats clustered to the different or same clusters at week 3 and week 12. Seven out of 10 rats shifted to different clusters (mixed-to-MS or MS-to-mixed) from week 3 to week 12. The agreement Kappa value for cluster classification at week 3 and week 12 was -0.4000 (95%CI: -0.9566 to 0.1566). This result indicates that although MS stress generated an isolated dysbiosis cluster in a fraction of rats, each rat's gut microbiota might shift between the less disturbed (mixed) cluster and the severely disturbed (MS) cluster.

UniFrac test on animal models and co-housing effect

We further tested the effect of modeling and co-housing on fecal microbiota using the weighted and unweighted UniFrac test (Table 2). A phylogenetic tree was built for all samples at each time point, and weighted and unweighted UniFrac scores were calculated to evaluate the community similarity. According to the unweighted UniFrac test, we found that by week 3, the fecal community in Ctl and MS groups was significantly different ($P < 0.001$). The co-housing effect caused community dissimilarity in the 2 houses of control rats (B8 *vs* C6, $P < 0.001$) but not in the 2 houses of MS rats (D3 *vs* G5, $P = 0.507$). By week 8, the co-housing effect was non-significant within the MS and Ctl groups, but the difference was still significant between these two groups. By week 12, a significant community difference existed among the Ctl, MS, and pTNBS groups; the co-housing effect was not obvious within any of the groups. The weighted UniFrac test was significant in all of the above comparisons. Overall, these data suggest that both the MS model and the pTNBS model developed dysbiosis of the fecal microbiota, and the differences were not caused by the co-housing relationship.


Week 3
Week 8
Week 12
Bacteroidetes
Proteobacteria
Firmicutes
Ctl
MS
MS_D
pTNBS

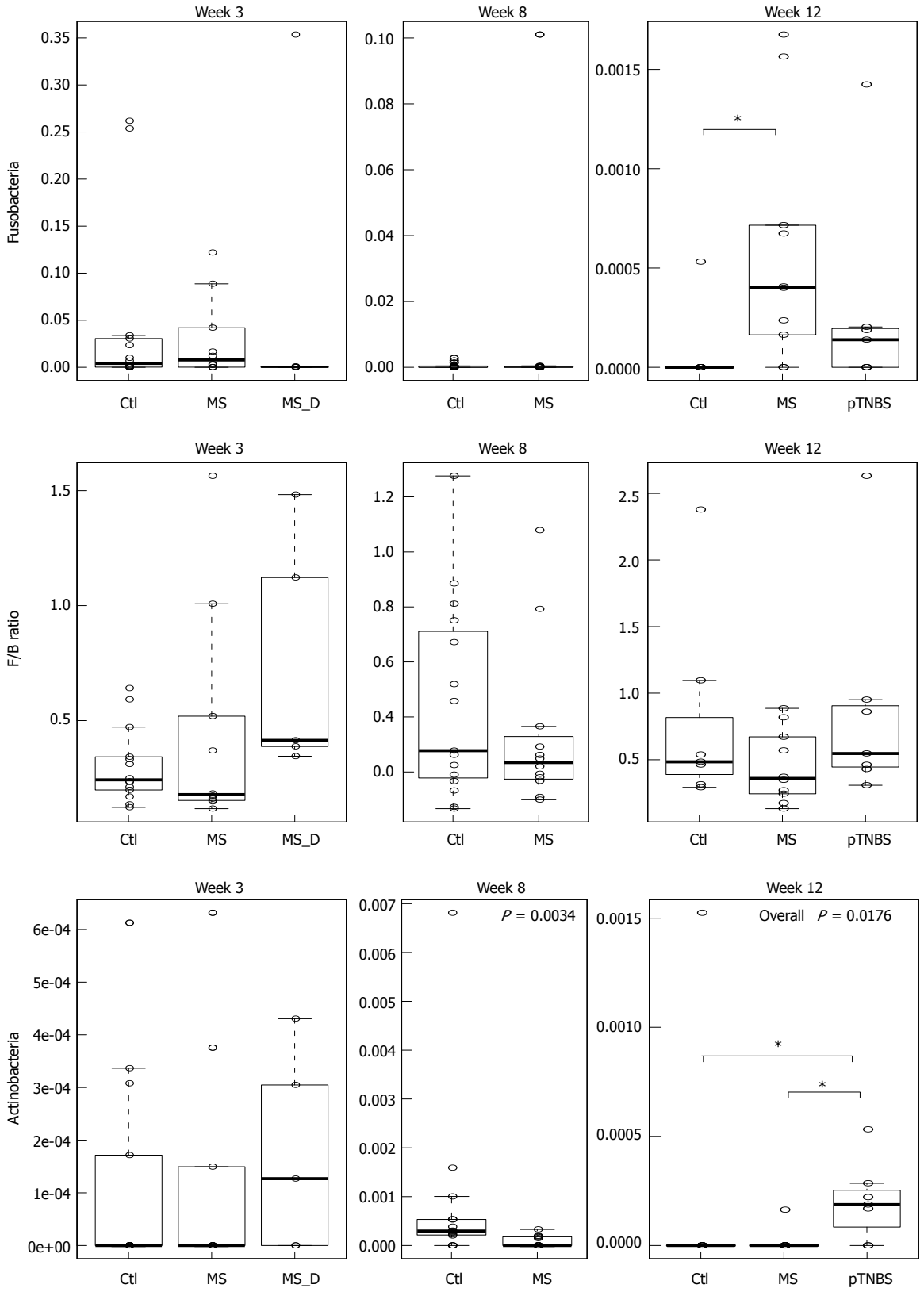


Figure 3 Phyla abundance by weeks 3, 8 and 12. Asterisk indicates $P < 0.05$ in pairwise comparison. $P > 0.05$ (no significant), unless the P-value is drawn in the plot box. MS: Maternal separation; MS_D: MS early death; pTNBS: TNBS post-inflammatory.

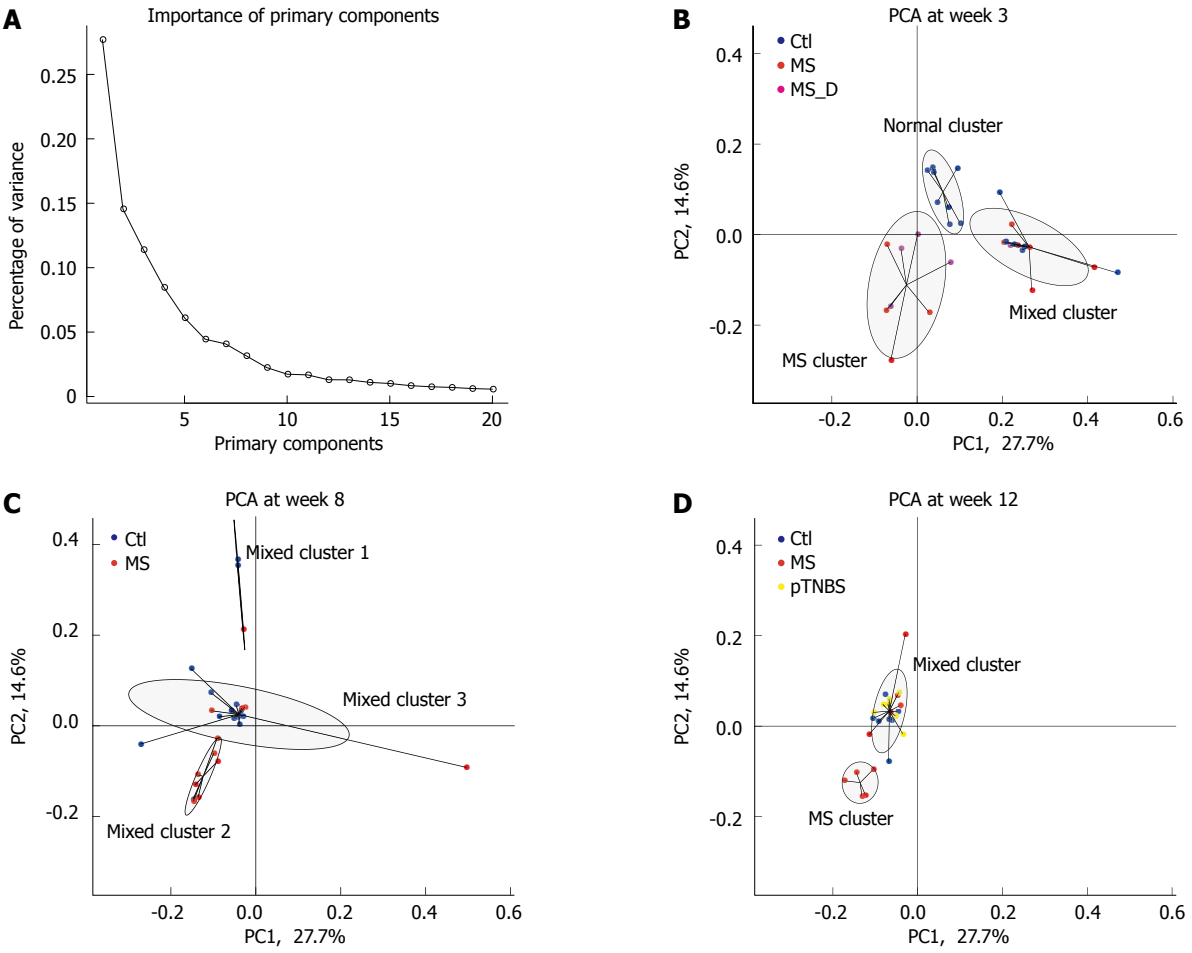


Figure 4 Primary component analysis and cluster analysis by time points. Primary components were calculated from the relative abundance of all 2413 OTUs. A: The percentage of variances explained by the first 20 primary components. The primary component was separately plotted at week 3 (B), week 8 (C), and week 12 (D). The cluster analysis divided the samples into three clusters, and the samples in the same cluster were connected together. The ellipse was estimated to cover 75% of dots in this cluster. Each cluster was named according to the samples involved in this cluster. The MS group formed isolated clusters, indicated as "MS cluster", at week 3 and week 12. MS: Maternal separation; PCA: Primary component analysis.

Table 1 Importance and meaning of the first 10 primary components of fecal microbiota

Primary component	% of variance	Difference among time points (n = 80, df = 2)		Difference among groups (n = 80, df = 3)	
		χ^2	*P*-value	χ^2	*P*-value
1	27.7%	37.67931	0.00000[1]	4.6999109	0.19514
2	14.6%	1.352165	0.50861	20.4278998	0.00014[1]
3	11.4%	6.165561	0.04583[1]	8.6830899	0.03382[1]
4	8.5%	0.719395	0.69789	21.7793862	0.00007[1]
5	6.1%	0.623716	0.73209	4.6400976	0.20013
6	4.5%	8.388254	0.01508[1]	9.3688439	0.02477[1]
7	4.1%	0.844768	0.65548	0.7871023	0.85255
8	3.2%	0.412918	0.81346	6.6021743	0.08572
9	2.2%	8.880049	0.01180[1]	9.9866961	0.01868[1]
10	1.7%	1.762893	0.41418	2.6274074	0.45270

[1]The *P*-value is less than 0.05.

Biomarkers and correlation to visceral hypersensitivity

We performed linear discriminant analysis coupled with effect size measurement (LEfSe) analysis at different time points to screen biomarkers for each group (Figure 5, Figure S1-3). By week 3, 29 samples were analyzed using LEfSe. The control group was strongly associated with higher abundances of unclassified *Bacteroidales*, *Veillonella*, *Treponema*, and unclassified *Clostridiales*. The MS group was associated with higher abundances of unclassified *Burkholderiales*, *Coprobacillus*, and *Clostridium_XIVa*. By week 8, 27 samples were analyzed. The MS group was associated with higher abundances of *Helicobacter*, unclassified *Burkholderiales*, and unclassified *Desulfovibrionaceae*,

Table 2 Weighted and unweighted UniFrac test on experiment groups and co-housing

Time point	Comparison	Number of samples to build phylogenetic tree	Weighted UniFrac test		Unweighted UniFrac test	
			Weighted UniFrac score	*P*-value	Unweighted UniFrac score	*P*-value
Week 3	Ctl-MS	29	0.863902	< 0.0010	0.957176	< 0.0010[1]
	Ctl-MS_D	29	0.926521	< 0.0010	0.963861	0.0130[1]
	MS-MS_D	29	0.833168	< 0.0010	0.909242	0.2790
	Ctl(B8)-Ctl(C6)	29	1.000000	< 0.0010	1.000000	< 0.0010[1]
	MS(D3)-MS(G5)	29	1.000000	0.0070	1.000000	0.5070
Week 8	Ctl-MS	27	0.719683	< 0.0010	0.951668	0.0190[1]
	Ctl(B8)-Ctl(C6)	27	0.83063	< 0.0010	0.955082	0.0870
	MS(D3)-MS(G5)	27	0.617715	< 0.0010	0.874196	0.5220
Week 12	Ctl-MS	24	0.754046	< 0.0010	0.944729	0.0390[1]
	Ctl-pTNBS	24	0.942882	< 0.0010	0.978698	0.0160[1]
	MS-pTNBS	24	0.828407	< 0.0010	0.973938	0.0130[1]
	Ctl(B4)-Ctl(C3)	24	0.928051	< 0.0010	0.976209	0.3200
	MS(D3)-MS(G5)	24	0.771421	< 0.0010	0.912219	0.4550
	pTNBS(B4)-pTNBS(C2)	24	1.000000	< 0.0010	1.000000	0.6390

[1]The *P*-value is less than 0.05. MS: Maternal separation; pTNBS: TNBS post-inflammatory.

which all belong to *Proteobacteria*. The control group was associated with higher abundances of *Barnesiella*, *Actinobacteria*, *Clostridium_XI*, *Allobaculum*, and *Odoribacter*.

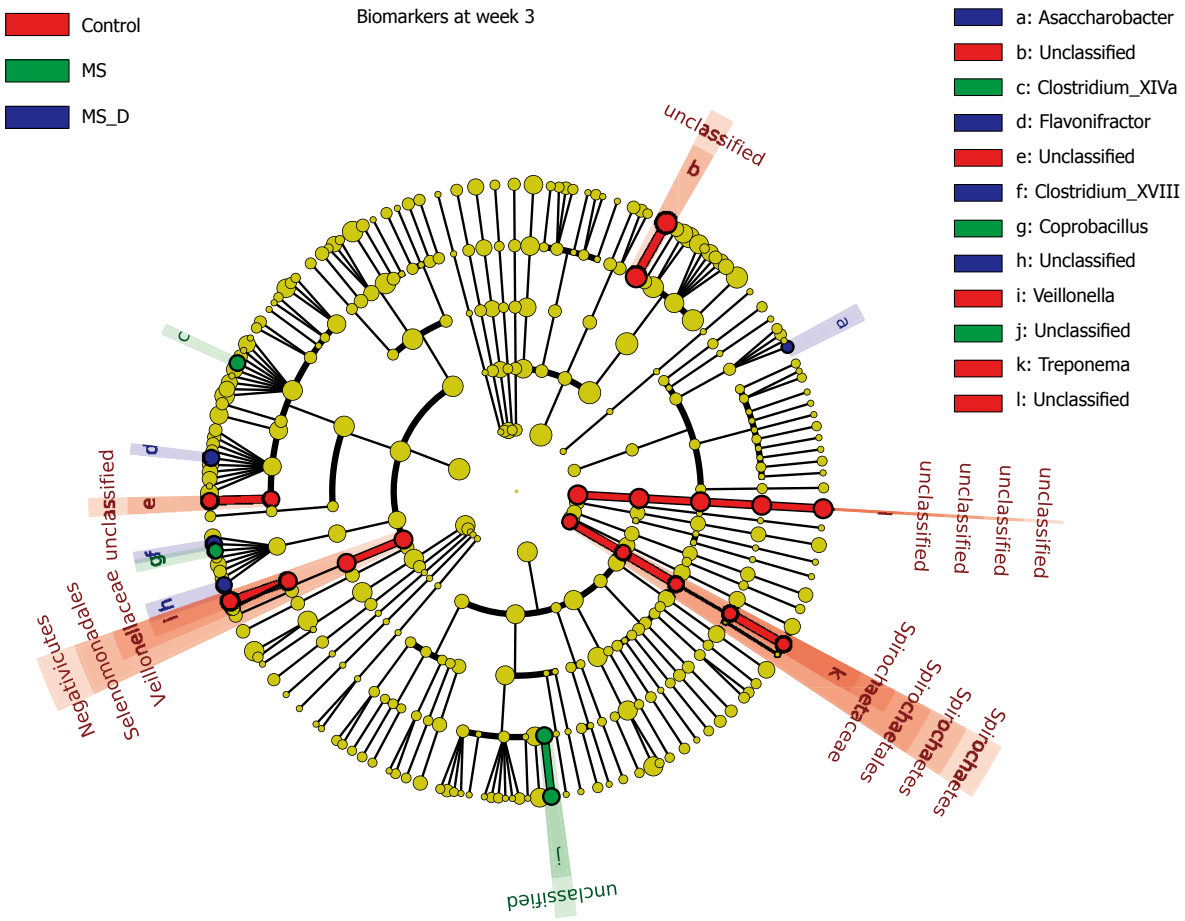
To investigate whether the differentially abundant taxa correlated with the visceral hypersensitivity level, we listed the biomarkers in each group by week 12, and calculated their Spearman correlation to the VHI both within the respective group and across groups (Table 3). By week 12, *Fusobacterium* was associated with the MS group (LDA Score = 2.766). The *Fusobacterium* abundance was also significantly and positively correlated with the VHI across the all 24 samples by week 12 (r = 0.4564, P = 0.0250). Unclassified *Erysipelotrichaceae* was associated with the control group (LDA Score = 3.097) and significantly and negatively correlated with the VHI across groups (r = -0.4944, P = 0.0140). These data suggest that *Fusobacterium* may participate in the pathogenesis of visceral hypersensitivity and that *Erysipelotrichaceae* might protect against the hypersensitivity.

Comparing visceral hypersensitive rat models to IBS patients

Next, we asked to what extent visceral hypersensitive rats' dysbiosis resembles that of IBS patients. We downloaded the pyrosequencing data published by Jeffery *et al*[6], which included 37 IBS patients and 20 controls. We used the LEfSe method to analyze disease and healthy biomarkers in human fecal samples (Figure 6A); this data was further compared to the data from our animal models (Figure 6B). We identified 36 biomarkers of IBS patients and 15 biomarkers of human controls[6] (Figure 6A). The biomarkers of disease were largely different in human IBS and visceral hypersensitive rats, and only a few disease or control biomarkers were shared between the human study[6] and our rat model study. *Fusobacteria* marginally increased in IBS patients compared with human controls[6] (P = 0.063, KW, Figure 6C). The MS rat model did not share increased common biomarkers with human patients. Both control rats and human health controls have fecal microbial markers of *Porphyromonadaceae* and unclassified *Porphyromonadaceae* (Figure 6B). In the IBS cohort published by Jeffery *et al*[6], *Porphyromonadaceae* was significantly lower in the IBS groups (P = 0.0006, KW, Figure 6D), and the MS rats also had lower *Porphyromonadaceae* concentrations (P = 0.0097, KW, Figure 6D). The genus *Clostridium_XI* (belonging to family *Peptostreptococcaceae*, order *Clostridiales*) was a biomarker of both IBS patients and pTNBS rats (Figure 6E). *Clostridium_XI* accounted for up to 6% of the fecal microbiota in the IBS group and was significantly higher than the level in the healthy control group (P = 0.0484, KW). Additionally, *Clostridium XI* also colonized at a higher level in the pTNBS rats (P = 0.0422, KW).

DISCUSSION

In this study, we found that (1) both the MS and the pTNBS rat models developed dysbiosis of fecal microbiota; (2) the fecal microbiota of the MS model was characterized by a lower diversity and a higher level of *Fusobacteria* at week 3 and week 12. A fraction of the MS rats formed an isolated MS cluster that indicated clear-cut dysbiosis in comparison to the controls but the rats in this cluster tended to alternate; (3) the pTNBS model was characterized by higher *Actinobacteria* but did not develop any isolated clusters; (4) among the biomarkers observed by week 12, the *Fusobacterium* positively and unclassified *Erysipelotrichaceae* negatively correlated to visceral hypersensitivity; and (5) in comparison to a previously published fecal microbial profile in human IBS patients[6], *Porphyromonadaceae* was a protective biomarker for both healthy humans and rat controls;


Control
MS
MS_D
Biomarkers at week 3
a: Asaccharobacter
b: Unclassified
c: Clostridium_XIVa
d: Flavonifractor
e: Unclassified
f: Clostridium_XVIII
g: Coprobacillus
h: Unclassified
i: Veillonella
j: Unclassified
k: Treponema
l: Unclassified
unclassified
Veillonellaceae
Selenomonadales
Negativicutes
Spirochaetaceae
Spirochaetales
Spirochaetes
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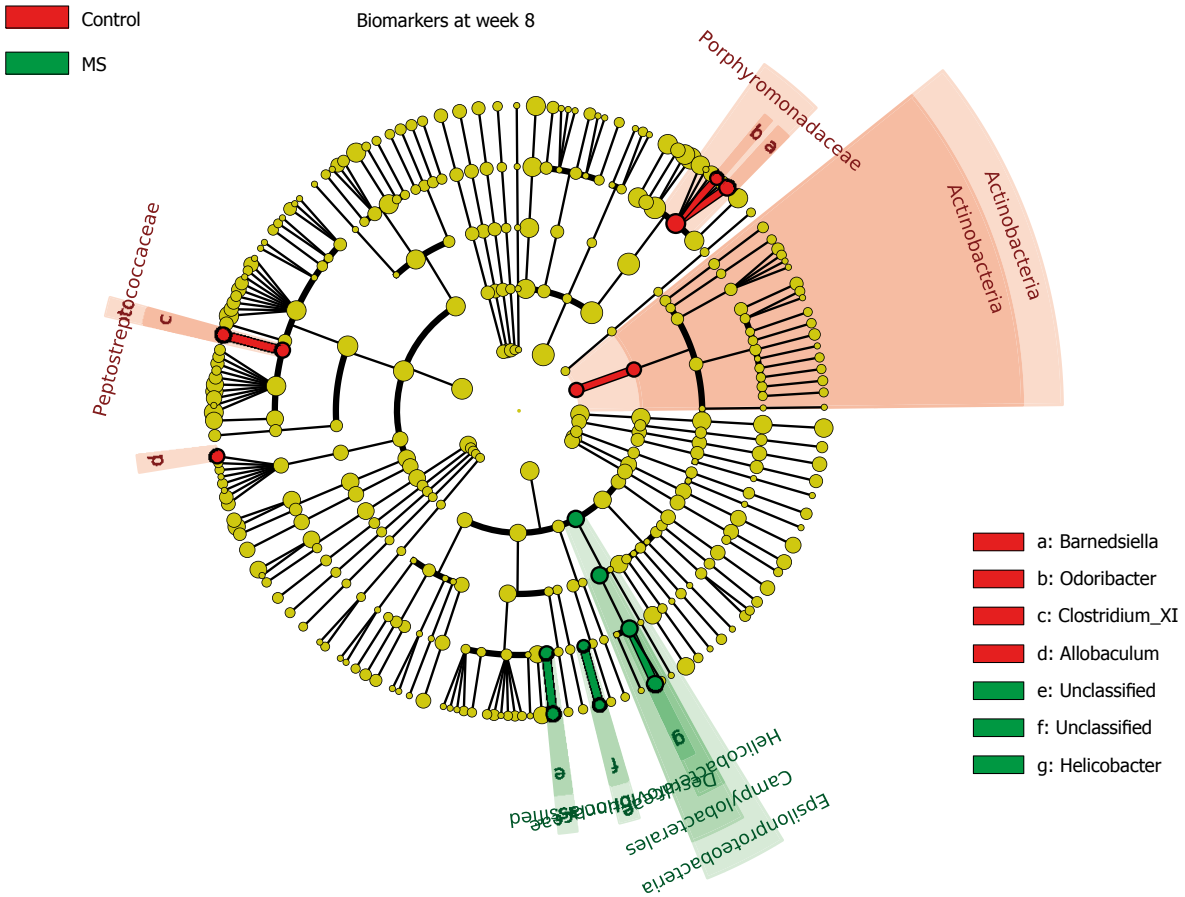

Control
MS
Biomarkers at week 8
a: Barnedsiella
b: Odoribacter
c: Clostridium_XI
d: Allobaculum
e: Unclassified
f: Unclassified
g: Helicobacter
Porphyromonadaceae
Actinobacteria
Peptostreptococcaceae
Helicobacteraceae
Campylobacterales
Epsilonproteobacteria

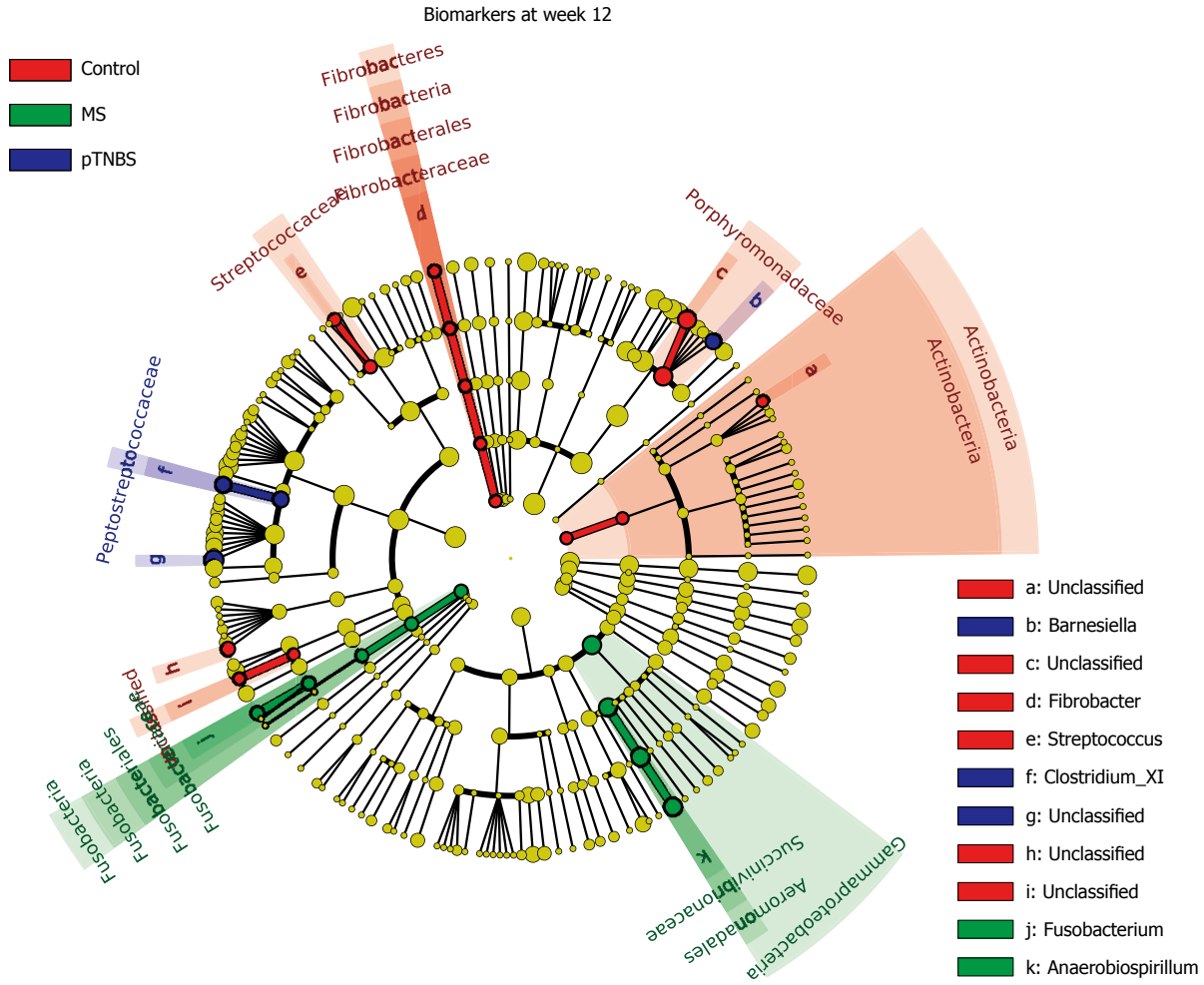


Figure 5 Microbial markers for different groups at weeks 3, 8 and 12. Biomarkers for each time point were calculated using the LEfSe Method. The abundances of taxa at the phylum, class, order, family, and genus levels were compared between the groups. Taxa with different abundances between groups and with an LDA score larger than 2.0 were considered to be a biomarker; biomarkers were indicated with corresponding colors on the cladogram. See also Figure S1, S2, and S3. MS: Maternal separation; MS_D: MS early death; pTNBS: TNBS post-inflammatory.

Clostridium_XI was a shared biomarker for human IBS patients and pTNBS rats.

Rodent models have been frequently used to study the pathogenesis and treatment of IBS, where the intestinal microbiota plays an important role. Before this study, dysbiosis in IBS rat models had not been specifically profiled, and whether any members in microbial community were correlated to visceral hypersensitivity was not known. We evaluated the fecal microbiota of MS and control rats at three time points and found that dysbiosis happened shortly after weaning (third week) and at the adult phase (12 wk). The pTNBS rat model did not develop an isolated microbial cluster but had biomarkers of *Barnesiella*, *Clostridium_XI*, and unclassified *Ruminococcaceae*. Through the unweighted UniFrac test, we found that the co-housing effect was no more significant at week 8 or week 12. Thus, both models developed significant dysbiosis of the fecal microbiota.

Approximately 10% of IBS patients believe that their symptoms began with an infectious illness, and prospective studies have shown that 3% to 36% of enteric infections lead to IBS symptoms[19]. Understanding underlying gut microbial dysbiosis associated with PI-IBS is critical for the prevention and management of this disease. *Campylobacter jejuni*, *Campylobacter rodentium*, and *Salmonella enterica* are available bacterial infectious murine models that mimic aspects of the pathogenesis of post-infectious IBS[8]. In this study, two rat models, MS and pTNBS, were not given any specific infector but were colonized more frequently by *Fusobacterium* and *Clostridium XI,* respectively. The latter includes the *Clostridium difficile*, *Clostridium litorale*, and *Clostridium lituseburense*. These two genera were also found to increase or tend to increase in the downloaded Miseq 16S rRNA gene sequencing data from Jeffery's IBS cohort[6]. Thus, these two bacteria may be common dysbiosis features across human and rat models. In a chip-based study by Jalanka-Tuovinen *et al*[7], *C. cellulosi* and its relatives (members from *Clostridium* cluster IV) significantly decreased in IBS-D patients. Thus, whether *Clostridium* plays a mechanistic role in the pathogenesis of IBS warrants further study.

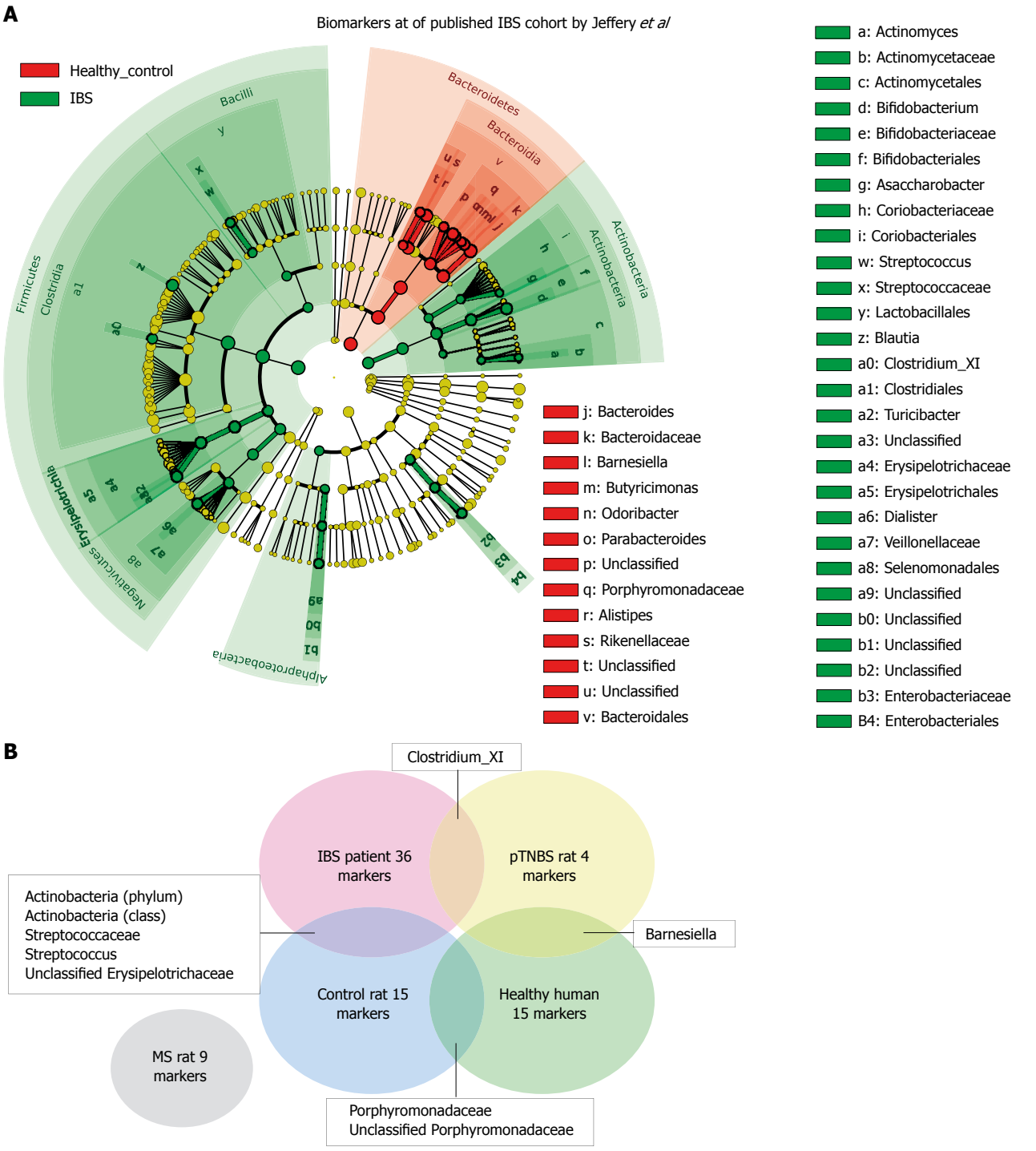
Table 3 Abundance of different taxa among groups by week 12 and correlations to visceral hypersensitivity

Taxonomy	LDA score (Log10)	Group	Across groups (n = 24)		Within group	
			r_all_sample	p_all_sample	r_ingroup	p_ingroup
Actinobacteria	2.338	Control n = 7	0.1835	0.3906	0.2041	0.6606
Actinobacteria | Actinobacteria	2.338		0.1835	0.3906	0.2041	0.6606
Actinobacteria | Actinobacteria | Coriobacteriales | Coriobacteriaceae | Unclassified	2.192		0.2639	0.2127	0.2041	0.6606
Bacteroidetes | Bacteroidia | Bacteroidales | Porphyromonadaceae	4.610		-0.1005	0.6405	0.1429	0.7825
Bacteroidetes | Bacteroidia | Bacteroidales | Porphyromonadaceae | Unclassified	4.543		-0.1344	0.5313	0.1429	0.7825
Fibrobacteres	2.435		-0.3601	0.0839	-0.0741	0.8745
Fibrobacteres | Fibrobacteria	2.435		-0.3601	0.0839	-0.0741	0.8745
Fibrobacteres | Fibrobacteria | Fibrobacterales	2.435		-0.3601	0.0839	-0.0741	0.8745
Fibrobacteres | Fibrobacteria | Fibrobacterales | Fibrobacteraceae	2.435		-0.3601	0.0839	-0.0741	0.8745
Fibrobacteres | Fibrobacteria | Fibrobacterales | Fibrobacteraceae | Fibrobacter	2.435		-0.3601	0.0839	-0.0741	0.8745
Firmicutes | Bacilli | Lactobacillales | Streptococcaceae	2.710		0.1215	0.5717	0.4447	0.3174
Firmicutes | Bacilli | Lactobacillales | Streptococcaceae | Streptococcus	2.710		0.1215	0.5717	0.4447	0.3174
Firmicutes | Erysipelotrichia | Erysipelotrichales | Erysipelotrichaceae | Unclassified	3.097		-0.4944	0.0140[1]	-0.1429	0.7825
Firmicutes | Negativicutes | Selenomonadales | Unclassified	2.396		-0.2047	0.3374	0.7027	0.0782
Firmicutes | Negativicutes | Selenomonadales | Unclassified | Unclassified	2.396		-0.2047	0.3374	0.7027	0.0782
Fusobacteria	2.766		0.4564	0.0250[1]	0.2067	0.5667
Fusobacteria | Fusobacteria	2.766		0.4564	0.0250[1]	0.2067	0.5667
Fusobacteria | Fusobacteria | Fusobacteriales	2.766		0.4564	0.0250[1]	0.2067	0.5667
Fusobacteria | Fusobacteria | Fusobacteriales | Fusobacteriaceae	2.766		0.4564	0.0250[1]	0.2067	0.5667
Fusobacteria | Fusobacteria | Fusobacteriales | Fusobacteriaceae | Fusobacterium	2.766	MS n = 10	0.4564	0.0250[1]	0.2067	0.5667
Proteobacteria | Gammaproteobacteria	4.622		0.1809	0.3976	0.0667	0.8648
Proteobacteria | Gammaproteobacteria | Aeromonadales	4.622		0.1757	0.4115	0.0667	0.8648
Proteobacteria | Gammaproteobacteria | Aeromonadales | Succinivibrionaceae	4.622		0.1757	0.4115	0.0667	0.8648
Proteobacteria | Gammaproteobacteria | Aeromonadales | Succinivibrionaceae | Anaerobiospirillum	4.622		0.1757	0.4115	0.0667	0.8648
Bacteroidetes | Bacteroidia | Bacteroidales | Porphyromonadaceae | Barnesiella	3.607		-0.1501	0.4840	-0.0180	0.9694
Firmicutes | Clostridia | Clostridiales | Peptostreptococcaceae	3.776	pTNBS n = 7	0.1946	0.3623	0.0180	0.9694
Firmicutes | Clostridia | Clostridiales | Peptostreptococcaceae | Clostridium_XI	3.776		0.1946	0.3623	0.0180	0.9694
Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | Unclassified	5.014		-0.0322	0.8813	-0.1982	0.6701

[1]The P-value is less than 0.05. LDA: Linear discriminant analysis; MS: Maternal separation; pTNBS: TNBS post-inflammatory.

By week 12, *Fusobacterium* colonized in significantly greater abundance in MS rats, and its abundance was positively correlated with higher VHI scores. The *Fusobacteria* phylum also tends to be higher in published IBS cohorts[6]. *Fusobacterium* was invasive to the gut epithelial cells and has already been documented as being involved in the pathogenesis of colorectal adenoma[20-22] and inflammatory bowel disease[23,24]. This represents the first study documenting that *Fusobacterium* was involved in visceral hypersensitivity. Whether the increased colonization of *Fusobacterium* caused low grade inflammation and thus contributed to visceral hypersensitivity is not currently known. Moreover, both *Fusobacterium* and members in *Clostridium* are known short chain fatty acid (SCFA) producers[24-26]. The low fermentable oligo-, di-, and monosaccharides and polyol (FODMAP) diet has been shown to be an efficacious therapy for the reduction of IBS symptoms in randomized controlled trials[27-29]. Supplementing food containing FODMAP to IBS patients and healthy people would trigger gastrointestinal symptoms to a larger extent in the patient group[30]. However, the mechanism of treatment with a low FODMAP diet and why IBS patients are more sensitive to FODMAP remains unknown[31]. In this study, we identified two butyric producing taxa, *Fusobacterium* and *Clostridium*, which significantly correlate to visceral hypersensitivity or are disease biomarkers. Their colonization may render patients more ready to produce SCFA and gas in the presence of FODMAP. It was reported that the butyrate-producing *Clostridium* cluster XIVa significantly increased in IBS patients consuming a typical high FODMAP diet compared with those on a low FODMAP diet[32]. Farmer *et al*[33] showed that caecal intraluminal pH was significantly lower in IBS patients compared to controls. Thus, the detailed mechanistic role of *Fusobacterium* and *Clostridium* in IBS warrants further study.

Porphyromonadaceae is a family belonging to the order of *Bacteroidales* and the class *Bacteroidetes*. Among others, *Barnesiella* and *Butyricimonas* are genera under *Porphyromonadaceae*. Unfortunately, the biological function of *Porphyromonadaceae* has not been characterized in detail, and little attention has been paid to their role in gastrointestinal diseases. A previous study documented that *Barnesiella* was enriched in dextran sulfate sodium-induced colitis[34]. In our study, *Barnesiella* was also enriched 4 weeks after TNBS instillation. Whether *Barnesiella* promoted the inflammation or was passively enriched under inflammatory conditions was not determined in the current study. A recent review paper[35] summarized 29 relevant original research articles concerning microbiota analysis and IBS. Durbán's pyrosequencing


A
Biomarkers at of published IBS cohort by Jeffery et al
Healthy_control
IBS
Bacilli
Bacteroidetes
Bacteroidia
Actinobacteria
Actinobacteria
Firmicutes
Clostridia
Negativicutes
Erysipelotrichia
Alphaproteobacteria
j: Bacteroides
k: Bacteroidaceae
l: Barnesiella
m: Butyricimonas
n: Odoribacter
o: Parabacteroides
p: Unclassified
q: Porphyromonadaceae
r: Alistipes
s: Rikenellaceae
t: Unclassified
u: Unclassified
v: Bacteroidales
a: Actinomyces
b: Actinomycetaceae
c: Actinomycetales
d: Bifidobacterium
e: Bifidobacteriaceae
f: Bifidobacteriales
g: Asaccharobacter
h: Coriobacteriaceae
i: Coriobacteriales
w: Streptococcus
x: Streptococcaceae
y: Lactobacillales
z: Blautia
a0: Clostridium_XI
a1: Clostridiales
a2: Turicibacter
a3: Unclassified
a4: Erysipelotrichaceae
a5: Erysipelotrichales
a6: Dialister
a7: Veillonellaceae
a8: Selenomonadales
a9: Unclassified
b0: Unclassified
b1: Unclassified
b2: Unclassified
b3: Enterobacteriaceae
B4: Enterobacteriales
B
Clostridium_XI
IBS patient 36 markers
pTNBS rat 4 markers
Actinobacteria (phylum)
Actinobacteria (class)
Streptococcaceae
Streptococcus
Unclassified Erysipelotrichaceae
Barnesiella
Control rat 15 markers
Healthy human 15 markers
MS rat 9 markers
Porphyromonadaceae
Unclassified Porphyromonadaceae
C
Fusobacteria
P = 0.063
0.00030
0.00025
0.00020
0.00015
0.00010
0.0005
0.0000
Healthy control
IBS

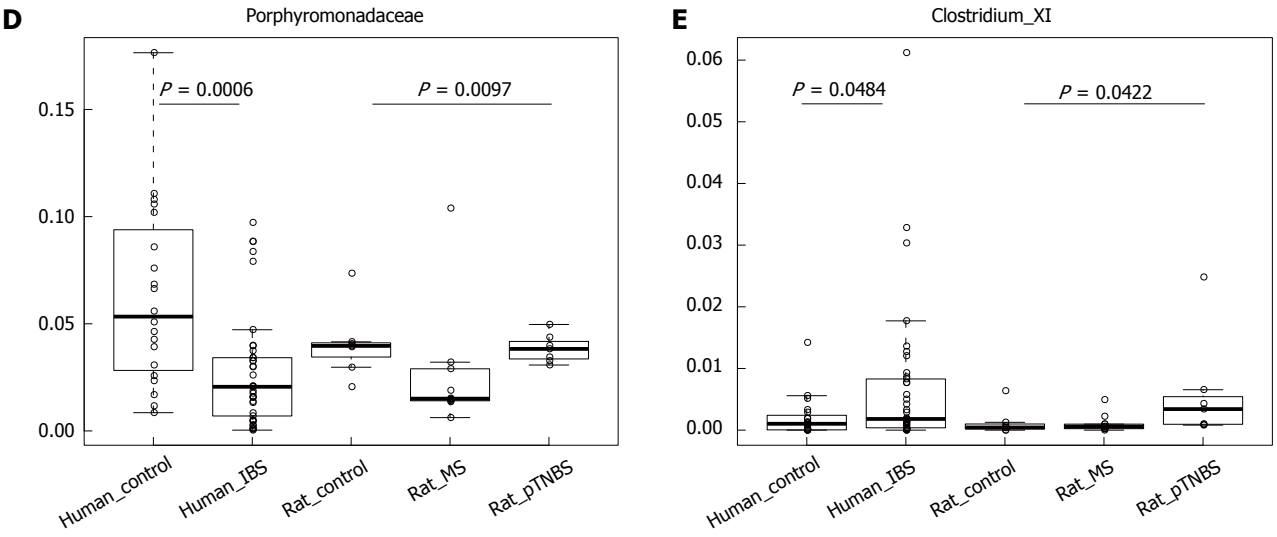


Figure 6 Comparing rat model dysbiosis to that of Jeffery's human irritable bowel syndrome cohort. A: Cladogram indicates the biomarkers of different abundances between groups; B: Venn plot of the positive (increased) biomarkers shared by rat models and Jeffery's human IBS cohort. The overlapped area indicates that the two groups have common biomarkers. The MS rat does not share biomarkers from the human cohort; C: Abundance of the *Fusobacteria* phylum marginally increased in the human IBS cohort; D: Abundance of *Porphyromonadaceae* in fecal samples of healthy human controls, human IBS patients, control rats, MS rats, and pTNBS rats. *Porphyromonadaceae* was depleted in both the human IBS group and the MS rats; E: Abundance of *Clostridium XI* in fecal samples of healthy human controls, healthy IBS patients, control rats, MS rats, and pTNBS rats. *Clostridium XI* increased in both human IBS patients and pTNBS rats. IBS: Irritable bowel syndrome; MS: Maternal separation; pTNBS: TNBS post-inflammatory.

study[36] found that the family *Porphyromonadaceae* was increased in the fecal samples of IBS subjects. In our study, the *Porphyromonadaceae* was highest in the control group by week 12. The discrepancy may be explained by the different nature between human patients and rat models.

IBS is a human disease with multifactorial pathophysiology[37], and the prevalence of IBS is associated with social-economic factors[38]. To date no available model could ideally model the IBS pathogenesis. IBS is heterogeneous and thus unlikely to be modeled in any single model. Although common biomarkers were found between human IBS patients and rat models, the limitations of rat models should also be taken into consideration. The pTNBS model was triggered by a pro-inflammatory molecule (TNBS). Therefore, this model resembles the human inflammatory bowel disease to some extent and can only mimic the post-infectious IBS, which is associated only to a percentage of patients. Furthermore, the causal relationship between visceral hypersensitivity, dysbiosis, and the symptoms of IBS is not clear and remains to be untangled in the future.

In summary, both the MS and the post-inflammation rat models developed dysbiosis in the fecal microbiota, and the models captured parts of the dysbiosis features of human IBS patients. The potential pathogenic role of *Fusobacterium* and *Clostridium XI*, as well as the protective role of *Porphyromonadaceae* warrants further mechanistic study.

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COMMENTS

Background

Previous studies have indicated that the gut microbiota participated in the pathogenesis of irritable bowel syndrome (IBS).

Research frontiers

Dysbiosis of the gastrointestinal microbiota and hypersensitivity to colonic distension are critical features of IBS. For animal models, the correlation between dysbiosis in the microbiota and visceral hypersensitivity remains unknown.

Innovations and breakthroughs

Dysbiosis triggered by neonatal maternal separation (MS) was lasting but not static. Both MS and post-inflammatory rat fecal microbiota deviated from that of the control rats to an extent that was larger than the co-housing effect. *Fusobacterium*, *Clostridium XI* and *Porphyromonadaceae* were identified as targets for future mechanistic research.

Applications

This study indicated that the two animal models could capture part of the dysbiosis features of IBS. Further mechanistic study on the biomarkers' role in the pathogenesis is warranted.

Peer-review

The manuscript is excellent and addresses adequately the relationship between dysbiosis and visceral hypersensitivity in experimental animals. The quality of the study design and experimental investigations are very high.

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ORIGINAL ARTICLE

Case Control Study

Factors affecting occurrence of gastric varioliform lesions: A case-control study

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Author contributions: Zou TH performed telephone interviews of most participants and wrote the manuscript; Zheng RH performed part of the telephone interviews; Gao QY and Kong X provided analytical tools; Chen XY offered the pathological data; Ge ZZ offered the endoscopic data; Chen YX served as scientific advisors; Fang JY designed the study and edited the manuscript; all authors approved the final version of the manuscript.

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Data sharing statement: Statistical code and dataset available from the corresponding author at jingyuanfang@sjtu.edu.cn.

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Abstract

AIM: To investigate the factors influencing the occurrence of gastric varioliform lesions (GVLs) and their possible link with gastric cancer.

METHODS: A 1:1 matched case-control study was performed to retrospectively analyze data from 1638 chronic gastritis patients who had undergone gastroscopy at one of two Chinese hospitals between 2009 and 2014. Patients with GVLs (cases) were compared to those without such lesions (controls). Endoscopic and pathological findings were recorded, along with interview information on *Helicobacter pylori* (*H. pylori*) infection, medical, drug and family histories, lifestyle and eating habits. The association between each factor and the occurrence of GVLs was estimated, and then multivariate conditional logistic regression was used to evaluate the independent factors.

RESULTS: The frequency and severity of glandular

atrophy, intestinal metaplasia (IM) and low-grade intraepithelial neoplasia were significantly increased in the GVL group ($P < 0.01$). Overall analysis showed that *H. pylori* infection [3.051 (2.157, 4.317), $P < 0.001$], allergic respiratory diseases [3.636 (2.183, 6.055), $P < 0.001$], work-related stress [2.019 (1.568, 2.600), $P < 0.001$], irregular meals [2.300 (1.462, 3.619), $P < 0.001$], high intake of spicy food [1.754 (1.227, 2.507), $P = 0.002$] and high intake of fresh fruit [0.231 (0.101, 0.529), $P = 0.001$] were significantly correlated with the occurrence of GVLs (positively, except for the latter). Stratified analyses indicated that pickled food consumption in patients over 50 years old [7.224 (2.360, 22.115), $P = 0.001$] and excessive smoking in men [2.013 (1.282, 3.163), $P = 0.002$] were also positively correlated, and that, for antral GVLs, vegetable consumption [0.491 (0.311, 0.776), $P = 0.002$] was negatively correlated.

CONCLUSION: Seven risk factors and two protective factors are determined for GVLs, which were found to be associated with premalignant abnormalities.

Key words: Gastric cancer; Gastric varioliform lesions; Precancerous lesion; Risk factor; Varioliform gastritis

Core tip: To our knowledge, this is the first case-control study investigating the factors influencing the formation of gastric varioliform lesions, which were supposed to be associated with gastric neoplasia in previous reports. Our results indicate a potentially increased cancer risk for the affected patients, and that *Helicobacter pylori* infection, allergic respiratory diseases, high work-related stress, irregular meals, high intake of spicy food, pickled food consumption in elder people, excessive smoking in men, consumption of vegetables and high intake of fresh fruit are found to be correlated with the occurrence of gastric varioliform lesions.

Zou TH, Zheng RH, Gao QY, Kong X, Chen XY, Ge ZZ, Chen YX, Zou XP, Fang JY. Factors affecting occurrence of gastric varioliform lesions: A case-control study. *World J Gastroenterol* 2016; 22(22): 5228-5236 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i22/5228.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i22.5228

INTRODUCTION

Varioliform gastritis (VG), or "octopus sucker" gastritis in the foreign literature and verrucous gastritis in the national literature, is a disease with a characteristic endoscopic manifestation but no specific clinical symptoms. The major endoscopic feature is the presence of gastric varioliform lesions (GVLs), namely, widespread small lesions, manifesting as round, oval or irregularly shaped elevations, often possessing a central umbilical-like depression covered in gray-colored secretion or tiny bleeds. In 1947, Moutier and Martin[1] first described two cases of this distinctive gastric mucosal disease, and then in 1978, Lambert *et al*[2] classified the disease, according to its site of occurrence, into "diffuse" VG when spread throughout the stomach, and "antral" VG when restricted to the antrum. These two forms of VG are thought to have different etiopathogenesis and histological manifestations[3]. VG has been recognized as a protruded type of chronic erosive gastritis in the Consensus on Chronic Gastritis in China (2012)[4], but endoscopists more often present the diagnosis as chronic gastritis with varioliform lesions.

Until recently, very little was known about the etiopathogenesis of GVLs. Malfertheiner *et al*[5] reported that the *Helicobacter pylori* (*H. pylori*) infection rate was 89% among 37 patients with GVLs, and their clinical symptoms and mucosal inflammation were substantially improved after effective eradication of the infection. In the national literature, most authors support this point of view and regard *H. pylori* as the main cause of GVLs. On the other hand, several studies have provided compelling evidence that type Ⅰ hypersensitivity may play a role[6]. Andre *et al*[7] found a large number of IgE-containing cells in the affected gastric mucosa and a significantly increased incidence of allergic diseases in patients with GVLs, as compared with the normal population. Furthermore, they performed a randomized double-blind placebo-controlled trial to compare clinical and endoscopic outcomes in patients treated with sodium cromoglycate, cimetidine or placebo[8]. The result stated that treatment with sodium cromoglycate greatly improved both sets of outcomes, whereas treatment with cimetidine or placebo showed no appreciable effect. Other previously reported pathogenic factors include hyperacid[9] and viral infection[10].

Some reports suggest a possible association between GVLs and gastric neoplasia. In 1960, Munoz Monteavaro *et al*[11] observed "*in situ*" carcinomatous transformation in a patient with VG, and other groups have reported similar findings more recently[12,13]. The elevated lesions can persist and transform into sessile polyps and appear as a gastric carcinoma several years later; as a result, the disease was classified as a precursor to gastric cancer at the World Congress of Gastroenterology (WCOG) in 1994. Diverse risk factors are involved in gastric carcinogenesis, including bacterial, environmental, dietary and genetic variables[14]. Numerous epidemiological studies have attempted to shed light on the factors impacting gastric neoplasia and precancerous lesions; these include a history of diabetes[15], aspirin consumption[16], excessive smoking[17] and drinking[18], pickled food consumption[19], tea consumption[20], amongst others. However, the results are somewhat inconsistent due

to the ethnic diversity and limited sample size. A recent systematic review concluded that smoking, drinking, red meat and pickled food were risk factors, and that fresh vegetables and fruit may be protective; there was insufficient evidence to draw conclusions regarding coffee, tea or seafood[21]. GVLs may share some of these risk factors, and clarifying the matter should provide a better understanding of this potentially premalignant condition, allowing physicians to better identify at-risk patients and to devise more effective treatment strategies. Therefore, we carried out a retrospective 1:1 matched bi-center case-control study, analyzing endoscopic and pathological data from 1638 patients with chronic gastritis. The association between potentially relevant variables and the occurrence of GVLs was systematically evaluated, with an aim to find independent risk factors and protective factors.

MATERIALS AND METHODS

Study sample and selection criteria

A 1:1 matched case-control study was conducted, analyzing data from outpatients who had undergone gastroscopy at Renji Hospital, Shanghai Jiao-Tong University School of Medicine or the Nanjing Drum Tower Hospital, Nanjing University School of Medicine between 2009 and 2014. A total of 1638 chronic gastritis patients were enrolled, all of which fell into one of two categories: those with GVLs (cases; n = 819) or those without such lesions (controls; n = 819).

To populate the case group, we searched the electronic databases of the aforementioned hospital endoscopic centers, using the following keywords: "varioliform gastritis" or "with gastric varioliform lesions" or "with erosive elevations"; then we closely examined the corresponding patient images and selected those patients having at least three typical lesions. Any disagreement was discussed by T.H. Zou and R.H. Zheng before reaching a consensus. Control patients, who were diagnosed with chronic gastritis at the same time, but without varioliform lesions, were matched one by one with the case group members, based on gender, age ± 2 years, month of examination and endoscopist. The exclusion criteria were strictly adhered to and were as follows: those who had no biopsy, those who were diagnosed with gastric cancer and those who had undergone partial or total gastrectomy. For those who had repeated examinations, we only recorded data from the first diagnostic gastroscopy.

Data extraction

The endoscopic and pathological findings were recorded. All the patients were required to undergo a gastroscopy with biopsies for the diagnosis. All parts of the upper gastrointestinal tract were carefully examined for any lesions by experienced endoscopists, and at least two biopsies were taken from the antrum. If suspected lesions were found, 2 to 5 more biopsies were taken. Pathological examinations for chronic gastritis were made by experienced pathologists according to the visual analogue scale (VAS) in the updated Sydney System[22,23] that is associated with the Consensus on Chronic Gastritis in China. Histological diagnosis of intraepithelial neoplasia was made based on the World Health Organization (WHO) classification[24]. To concretely differentiate the severity of inflammation, glandular atrophy or IM in the present study, a scheme was introduced using the following calculation: grading index = $(S_1 \times B_1 + S_2 \times B_2 + \ldots + S_n \times B_n)/B_n$, where S is the severity of a particular biopsy specimen, B is the number of the relevant specimen and n is the quantity of specimens[25].

H. pylori infection was detected using a *H. pylori* rapid urease test during endoscopic examination, HE and Giemsa staining of biopsy specimens, and a ^{13}C urea breath test. We defined a positive result as meeting one of the following two criteria: (1) the rapid urease test or HE staining was positive; or (2) if both urease and HE results were negative, yet the specimen was highly inflamed, Giemsa staining was added or a ^{13}C urea breath test was performed, and a positive outcome was considered indication of *H. pylori* infection. A ^{13}C urea breath test was subsequently used when evaluating the effect of eradication on *H. pylori* infection.

A questionnaire was designed by the authors and it was used to conduct telephone interviews with all patients in the study. The investigators were trained to be polite and methodical during interviews and they avoided calling patients at working, or otherwise busy hours. The questionnaire requested information on the patient's gender, age, *H. pylori* infection history, medical history, allergic diseases, drug history, family history, long-term lifestyle and eating habits. *H. pylori* infection history was categorized according to four different conditions: currently infected, but with no previous history of infection; chronic (repeated or persistent) infection; past infection that has been completely eradicated; no current or past infection. Allergic diseases consisted of bronchial asthma, allergic rhinitis, allergic skin disease, drug allergy, *etc*. The presence of allergic diseases was mainly based on the interview data, and the authors made the judgment with reference to the guideline of diagnosis for each disease. Lifestyle variables included sleep quality, work-related stress, tobacco smoking and alcohol consumption. Eating habits comprised irregular meals, intake of spicy food, pickled food, fried food, fresh fruit and vegetables; consumption of a particular food type over four time per week was considered high.

Statistical analysis

Statistical analyses were performed using SPSS Version 20.0 (SPSS Inc., Chicago, IL, United States).

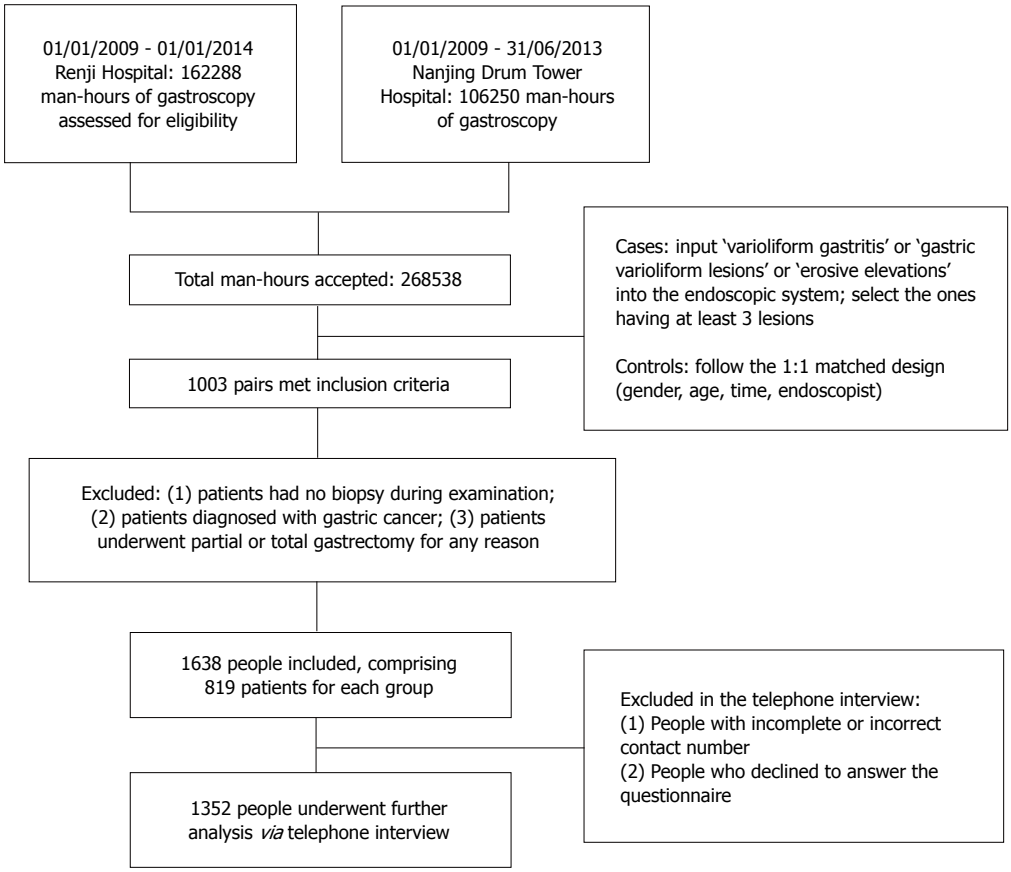

Figure 1 Flow chart of participant selection in the case-control study.

Table 1 Characteristics of the case group *n* (%)

Characteristic	
In total	819 (100)
Hospital	
Renji	491 (60.0)
Drum tower	328 (40.0)
Age (yr)	
< 50	278 (33.9)
⩾ 50, < 60	289 (35.3)
⩾ 60	252 (30.8)
Gender	
Men	448 (54.7)
Women	371 (45.3)
Illness part	
Antral form	806 (98.4)
Diffuse form	13 (1.6)
H. pylori infection	
H. pylori (+)	263 (32.1)
H. pylori (-)	556 (67.9)
Histology	
Glandular atrophy	363 (44.3)
IM	265 (32.4)
Intraepithelial neoplasia	92 (11.2)

H. pylori: *Helicobacter pylori*.

Measurement data were compared between the two groups using a paired *t*-test; where appropriate, numerical data were subjected to a chi-square test, while categorical data using a Mann-Whitney test. Single-factor analysis was used to estimate the association between each potential factor and GVLs, and then multivariate conditional Logistic regression analysis was applied to determine the independent risk and protective factors. Odds ratios (ORs) with 95% confidence intervals (95%CIs) were used to assess the magnitude of the associations. A two-sided *P*-value < 0.05 was considered statistically significant.

RESULTS

The systematic database search yielded 268538 man-hours of gastroscopy over the five-year period of interest. Following the inclusion/exclusion criteria and the 1:1 matched design, 1638 subjects were selected to populate the case and control groups. A flow chart of the selection process is presented in Figure 1. There were 448 men and 371 women in the case group, with a mean age of 53.40 ± 11.41 years (ranging from 18 to 87 years old). The basic, endoscopic and pathological characteristics of the case group are shown in Table 1.

Analysis of histological data

We compared the frequencies of *H. pylori* infection, glandular atrophy, IM and intraepithelial neoplasia between the cases and controls using a χ^2 test. The difference was significant for *H. pylori* infection (OR

Table 2 Overall and stratified analyses of histological data in the case-control study

	Case	Control	OR [95%CI]	*P*-value
Overall analysis				
H. pylori (+)	263	141	2.275 [1.801, 2.872]	< 0.001[b]
Glandular atrophy	363	271	1.610 [1.317, 1.967]	< 0.001[b]
IM	265	182	1.674 [1.343, 2.087]	< 0.001[b]
Intraepithelial neoplasia	92	65	1.468 [1.052, 2.049]	0.023[a]
Antral form				
H. pylori (+)	254	139	2.208 [1.745, 2.794]	< 0.001[b]
Glandular atrophy	341	268	1.472 [1.202, 1.803]	< 0.001[b]
IM	409	179	3.609 [2.908, 4.479]	< 0.001[b]
Intraepithelial neoplasia	79	65	1.239 [0.878, 1.747]	0.222
Diffuse form				
H. pylori (+)	9	2	12.375 [1.828, 83.767]	0.015[a]
Glandular atrophy	13	3		< 0.001
IM	11	3		0.005
Intraepithelial neoplasia	12	0	18.333 [2.522, 133.26]	< 0.001[b]

[a]$P < 0.05$; [b]$P < 0.01$, the case group *vs* the control group. *H. pylori*: *Helicobacter pylori*.

= 2.275, 95%CI: 1.801-2.872, $P < 0.01$), especially in the patients with diffuse GVLs. We also noted statistically significant findings in the pooled analysis for the association between glandular atrophy, with or without IM, and the formation of such lesions. Furthermore, a significantly increased risk of low-grade intraepithelial neoplasia was observed in the case group (OR = 1.468, 95%CI: 1.052-2.049, P = 0.023); this is known to be a premalignant condition. When the patients were further stratified according to VG form, the differences between patients with antral lesions and their matched controls were significant for glandular atrophy and IM, but not for intraepithelial neoplasia. On the other hand, diffuse lesions were strongly associated with all these histological parameters, including intraepithelial neoplasia. The results of the overall and stratified analyses are presented in Table 2.

Indices for grading inflammation, glandular atrophy and IM were calculated separately for the GVL patients and their matched controls. Paired *t*-tests showed that all these indices were significantly increased in the case group ($P < 0.01$).

Analysis of telephone interview data

For 49 patients, the contact number was incomplete or incorrect, and 94 declined to answer the questionnaire. Thus, there was a total of 1352 participants, comprising 676 people from each group; the answering ratio was 82.5%. Single-factor analyses of potential factors demonstrated that current infection with *H. pylori* (OR = 2.203), chronic infection with *H. pylori* (OR = 2.493), bronchial asthma (OR = 6.837), allergic rhinitis (OR = 2.963), family history of gastric cancer (OR = 1.926), high work-related stress (OR = 1.871), irregular meals (OR = 1.703), and high intake of spicy food (OR = 1.540) were positively associated with the occurrence of GVLs, while high intake of fresh fruit was negatively associated (OR = 0.721).

We found a negative correlation between current and chronic infection with *H. pylori* (Pearson coefficient, -0.113), and a positive correlation between bronchial asthma and allergic rhinitis (Pearson coefficient, 0.151). No correlation was found for any other factors. For subsequent analyses, we combined current and chronic infection into a single "*H. pylori* infection" category, and asthma and rhinitis were combined into "allergic respiratory diseases". Based on the results of single-factor analyses, we included the factors with a *P*-value less than 0.05 into the multivariate conditional Logistic regression equation. The adjusted analysis suggested that *H. pylori* infection, allergic respiratory diseases, high work-related stress, irregular meals and high intake of spicy food were independent risk factors for the formation of GVLs; and that high intake of fresh fruit was an independent protective factor. Table 3 shows the overall results of the single-factor and multivariate analyses.

Stratified analysis of telephone interview data

The participants were stratified by age, gender and VG form, and the results of the subsequent analysis are shown in Table 4. For those under 50 years old, high intake of fried food was significantly more common in the GVL group (P = 0.038) under univariate analysis; however, the correlation was not significant in the final multivariate analysis, suggesting that fried food intake may be a confounding factor. In those $\geqslant$ 50 years old, univariate and multivariate analyses indicated that pickled food consumption was a new independent risk factor for GVLs. In males, excessive smoking was also found to be a new independent risk factor, while in females, allergic skin diseases seemed to be a confounding factor. For antral form, single-factor analyses showed significant differences between cases and controls for fried food consumption and intake of vegetable side dishes, but only the latter factor was confirmed as an independent factor by the adjusted multivariate analysis. For diffuse form, current or chronic *H. pylori* infection was found in more than half

Table 3 Overall single-factor and multivariate analyses of impact factors in the case-control study

Impact factor	Univariate analysis			Multivariate analysis		
	OR	95%CI	*P*-value	OR	95%CI	*P*-value
H. pylori infection	2.329	[1.802, 3.011]	< 0.001[b]	3.051	[2.157, 4.317]	< 0.001[b]
Allergic Res. Dis.	3.745	[2.365, 5.930]	< 0.001[b]	3.636	[2.183, 6.055]	< 0.001[b]
Family history of GC	1.926	[1.059, 3.503]	0.029[a]	1.628	[0.801, 3.309]	0.178
Stress ↑	1.871	[1.344, 2.603]	< 0.001[b]	2.019	[1.568, 2.600]	< 0.001[b]
Irregular meals	1.703	[1.184, 2.449]	0.004[b]	2.300	[1.462, 3.619]	< 0.001[b]
Spicy food ↑	1.540	[1.156, 2.052]	0.003[b]	1.754	[1.227, 2.507]	0.002[b]
Fresh fruit ↑	0.721	[0.533, 0.974]	0.033[a]	0.231	[0.101, 0.529]	0.001[b]

[a]$P < 0.05$; [b]$P < 0.01$, the case group *vs* the control group. *H. pylori*: *Helicobacter pylori*.

of the affected patients, whereas only two matched controls had ever been infected. The diffuse form seemed to be more highly correlated with *H. pylori* infection, but with only thirteen pairs of participants making up the sample, no more than a general tendency could be assessed. Allergic respiratory diseases and a family history of gastric cancer were more frequent in patients with diffuse varioliform lesions *vs* matched controls.

DISCUSSION

As is widely accepted, intestinal-type gastric carcinogenesis is a multi-stage process, developing from chronic gastritis through a series of precancerous abnormalities to gastric carcinoma[26,27]. In addition, *H. pylori* infection is thought to be the key promoter[28,29]. These precursor conditions include chronic atrophic gastritis with or without IM, with a reported malignancy rate of 0.5%-1%[30,31], and intraepithelial neoplasia[32], which is classified from low to high grade according to WHO specifications. It is reported that 0%-15% of low-grade intraepithelial neoplasia could progress to high-grade, which has an extremely high malignancy rate of 25%-85%[33]. In the present study, the frequency and severity of glandular atrophy, IM and low-grade intraepithelial neoplasia were significantly elevated in the case group, indicating that the presence of GVLs is a potential risk factor for cancer. Nevertheless, no high-grade intraepithelial neoplasia was observed, and the results were inconsistent when analysis was restricted to antral varioliform lesions. Thus, this malignancy risk should be further investigated *via* a large-scale prospective study.

In view of the association between GVLs and *H. pylori* infection status, the literature is somewhat inconclusive[34]. Our analysis showed a statistically significant difference in infection rates between GVL patients (32.1%) and controls (17.2%). The adjusted analysis of the interview data indicated that *H. pylori* infection, especially chronic persistent infection, was a pathogenic factor. In contrast, no correlation existed where infections had been successfully treated. Thus, *H. pylori* eradication and regular endoscopic follow-ups should be key components of the treatment for GVLs.

European researchers have suggested that there may be an allergic component in the pathogenesis of the disease, specifically that excessive histamine release could play a central role[7,35]; however, no evidence for this has been reported for Chinese patients. Interestingly, we found that the frequency of allergic diseases was increased in patients with varioliform lesions, in particular bronchial asthma and allergic rhinitis. There were 112 GVL patients (16.6%) with at least one allergic disease, and the multivariate analysis confirmed that allergic respiratory disease was an independent risk factor. Family history of gastric carcinoma has been reported as a risk factor for gastric carcinogenesis[36,37], but it was not associated with GVLs in our study. Diffuse form appeared to have a more positive association with allergic diseases and family history of gastric cancer, yet the results were not conclusive owing to the limited sample size, and will thus need to be verified by larger studies in the future.

In the pooled multivariate analysis, the independent risk factors were high work-related stress, irregular meals, and high intake of spicy food, and the one potentially protective factor was high intake of fresh fruit. The stratified analyses indicated that pickled food consumption in people over 50 years old and excessive smoking in men were also risk factors. Intake of vegetable side dishes was found to be negatively correlated with the antral form of GVLs. Indeed, certain habits of daily life could serve as important risk factors for gastric cancer. Previous studies revealed a close association between negative psychological factors like nervousness or anxiety and susceptibility to neoplasia[38,39]. Our participants with high work-related stress could have an increased risk of gastric malignancy, which may be related to constant anxiety-induced stimulation of the sympathetic system. Smoking is also considered a pathogenic factor for multiple cancers. A 50-year observational study of 34439 British doctors indicated that cigarette smoking was a risk factor in the progression of 14 different cancers including gastric carcinoma[40]. In the present study, excessive smoking in men contributed significantly to the risk of GVLs, but not in women, indicating possible male predominance in the morbidity

Table 4 Stratified single-factor and multivariate analyses of impact factors in the case-control study

Factor	Case	Control	Univariate analysis		Multivariate analysis	
			OR [95%CI]	*P*-value	OR [95%CI]	*P*-value
Age < 50 yr						
H. pylori infection	69	37	2.224 [1.419, 3.487]	< 0.001[b]	1.968 [1.222, 3.170]	0.005[b]
Allergic Res. Dis.	34	11	3.445 [1.700, 6.978]	< 0.001[b]	3.784 [1.715, 8.347]	0.001[b]
Stress ↑	41	24	1.858 [1.083, 3.189]	0.023[a]	1.452 [1.076, 1.960]	0.015[a]
Irregular meals	40	25	1.723 [1.008, 2.946]	0.045[a]	2.207 [1.112, 4.381]	0.024[a]
Fried food ↑	56	38	1.622 [1.025, 2.567]	0.038[a]	1.459 [0.846, 2.517]	0.174
Spicy food ↑	50	33	1.654 [1.021, 2.681]	0.040[a]	1.838 [1.011, 3.342]	0.046[a]
Age ≥ 50 yr						
H. pylori infection	151	79	2.386 [1.745, 3.263]	< 0.001[b]	3.402 [2.149, 5.386]	< 0.001[b]
Allergic Res. Dis.	51	14	3.988 [2.173, 7.319]	< 0.001[b]	4.894 [2.164, 11.069]	< 0.001[b]
Stress ↑	68	39	1.879 [1.237, 2.855]	0.003[b]	2.265 [1.594, 3.219]	< 0.001[b]
Irregular meals	44	27	1.699 [1.032, 2.798]	0.035[a]	1.680 [0.918, 3.074]	0.092
Picked-food cons.	149	122	1.334 [1.001, 1.778]	0.049[a]	7.224 [2.360, 22.115]	0.001[b]
Spicy food ↑	86	62	1.481 [1.036, 2.117]	0.031[a]	1.786 [1.114, 2.863]	0.016[a]
Fresh fruit ↑	387	405	0.637 [0.409, 0.993]	0.045[a]	0.422 [0.178, 1.001]	0.050
Male						
H. pylori infection	116	67	2.054 [1.458, 2.893]	< 0.001[b]	3.445 [2.114, 5.612]	< 0.001[b]
Allergic Res. Dis.	42	10	4.599 [2.272, 9.310]	< 0.001[b]	6.563 [2.832, 15.209]	< 0.001[b]
Smoking	99	76	1.410 [1.003, 1.981]	0.047[a]	2.013 [1.282, 3.163]	0.002[b]
Stress ↑	63	34	2.023 [1.298, 3.154]	0.002[b]	2.096 [1.489, 2.950]	< 0.001[b]
Irregular meals	52	34	1.614 [1.021, 2.551]	0.039[a]	2.201 [1.262, 3.839]	0.005[b]
Spicy food ↑	79	57	1.488 [1.022, 2.165]	0.037[a]	2.167 [1.285, 3.653]	0.004[b]
Female						
H. pylori infection	104	49	2.727 [1.850, 4.021]	< 0.001[b]	3.031 [1.897, 4.844]	< 0.001[b]
Allergic Res. Dis.	43	15	3.183 [1.726, 5.867]	< 0.001[b]	3.502 [1.691, 7.255]	0.001[b]
Allergic skin Dis.	15	5	3.106 [1.114, 8.660]	0.023[a]	3.223 [0.966, 10.748]	0.057
Stress ↑	46	29	1.694 [1.032, 2.780]	0.036[a]	1.873 [1.356, 2.589]	< 0.001[b]
Irregular meals	32	18	1.872 [1.026, 3.415]	0.039[a]	2.027 [0.905, 4.538]	0.086
Spicy food ↑	57	38	1.619 [1.036, 2.530]	0.033[a]	2.185 [1.236, 3.861]	0.007[b]
Antral form						
H. pylori infection	211	114	2.248 [1.734, 2.914]	< 0.001[b]	3.124 [2.192, 4.452]	< 0.001[b]
Allergic Res. Dis.	80	25	3.552 [2.237, 5.639]	< 0.001[b]	3.432 [2.062, 5.712]	< 0.001[b]
Stress ↑	107	63	1.833 [1.315, 2.554]	< 0.001[b]	1.984 [1.544, 2.550]	< 0.001[b]
Irregular meals	83	52	1.681 [1.168, 2.422]	0.005[b]	2.191 [1.407, 3.412]	0.001[b]
Fried food cons.	202	169	1.281 [1.007, 1.629]	0.044[a]	1.338 [0.955, 1.876]	0.091
Spicy food ↑	133	95	1.500 [1.124, 2.002]	0.006[b]	1.705 [1.188, 2.447]	0.004[b]
Vegetable Cons.	227	262	0.797 [0.637, 0.996]	0.046[a]	0.491 [0.311, 0.776]	0.002[b]

[a]$P < 0.05$; [b]$P < 0.01$, the case group *vs* the control group. *H. pylori*: *Helicobacter pylori*.

of the disease. Concerning food consumption, pickled foods have been associated with the development of esophageal and gastric cancers, which can damage gastric mucosa and exacerbate the inflammation caused by *H. pylori*[41]. In recent times, Chinese dietary habits have changed dramatically. Pickled food may now be less popular in younger sections of the population, whereas spicy foods have greatly increased in popularity. Although capsaicin in spicy food has been shown to help counter the growth of *H. pylori*[42], we found that high intake of spicy food was a risk factor for varioliform lesions. The reason could be related to oncogene exposure or a chemical process during production. Meanwhile, the present study also provided factors that potentially offer some protection against GVLs. Intake of fresh vegetables and fruit has been reported to beneficial for avoidance of gastric neoplasia[43], which is consistent with the corresponding reduction in the frequency of GVLs seen in our study.

Several limitations of the present study must be taken into account. First, it is a retrospective analysis, for which recall bias and selection bias cannot be completely removed; a prospective study would be required to establish a convincing causal relationship between the factors and the disease. Second, the conclusions of the stratified analyses may be of limited value because of the small sample size, especially in the diffuse form group. Thus, some of the results in our study should be interpreted cautiously. Third, other relevant variables such as body mass index (BMI), hyperlipidemia, ABO blood group, consumption of coffee, carbonated drinks and bean products were not included; in addition, several factors such as the type of cigarette or alcohol consumed, medication dose and professional mental scale were not precisely classified. If the above factors were included in the multivariate regression equation, our results could have been very different. Future studies should therefore use a more complete set of variables.

To the best of our knowledge, this is the first case-control study investigating the factors influencing the formation of GVLs. The results suggest a potentially increased cancer risk for the affected patients, and that *H. pylori* infection, allergic respiratory diseases, high work-related stress, irregular meals, high intake of spicy food, pickled food consumption in older people, and excessive smoking in men were all positively correlated with the occurrence of GVLs. In contrast, consumption of vegetables and high intake of fresh fruit were found to be negatively correlated and therefore potentially protective. In summary, our results suggest that formation of GVLs can be reduced by maintaining a healthy lifestyle and positive attitude, while ensuring that allergic diseases and *H. pylori* infection are treated effectively. We suggest that a prospective study should be carried out in the future to examine the morphological and pathological evolution of GVLs, and thereby clarify their relationship with gastric malignancy. A large-scale, well-designed clinical trial is also warranted to provide more precise and robust conclusions on this matter.

COMMENTS

Background

Researchers discovered the presence of gastric varioliform lesions (GVLs) over 60 years ago, but until now, very little was known about the etiopathogenesis and progression. So the authors try to provide a better understanding of this potentially premalignant disease in the present case-control study.

Research frontiers

Previous reports suggested a possible association between GVLs and gastric cancer. And the disease was classified as a precursor to gastric cancer at the World Congress of Gastroenterology in 1994. More recently, Zhang *et al* performed a proteomic analysis to provide more molecular biological details of GVLs. The important differential proteins could serve as potential biomarkers for the early diagnosis of gastric cancer.

Innovations and breakthroughs

This is the first case-control study investigating the factors influencing the formation of GVLs, and the manuscript provide a better understanding of this potentially premalignant condition, allowing physicians to better identify at-risk patients and to devise more effective treatment strategies.

Applications

A large-scale, well-designed prospective study should be carried out in the future to examine the morphological and pathological evolution of GVLs, and thereby clarify their relationship with gastric malignancy.

Terminology

The term GVLs in the present study is a synonym of varioliform gastritis. The major endoscopic feature of such lesions is widespread small lesions, possessing a central umbilical-like depression covered in gray-colored secretion or tiny bleeds. Patients in China are affected more often by the antral type of the disease, thus endoscopists present the diagnosis as GVLs.

Peer-review

Patients diagnosed in an early stage of gastric cancer present an excellent prognosis, with a five-year survival rate greater than 90%. This well conducted and written retrospective case-control study considers the different risk and protective factors influencing the occurrence of GVLs and their possible link with development of gastric cancer.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Long-term outcomes and prognostic factors of patients with obstructive colorectal cancer: A multicenter retrospective cohort study

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Abstract

AIM: To investigate the long-term oncologic outcomes and prognostic factors in patients with obstructive colorectal cancer (CRC) at multiple Japanese institutions.

METHODS: We identified 362 patients diagnosed with obstructive colorectal cancer from January 1, 2002 to December 31, 2012 in Yokohama Clinical Oncology Group's department of gastroenterological surgery. Among them, 234 patients with stage Ⅱ/Ⅲ disease who had undergone surgical resection of their primary lesions were analyzed, retrospectively. We report the long-term outcomes, the risk factors for recurrence, and the prognostic factors.

RESULTS: The five-year disease free survival and cancer-specific survival were 50.6% and 80.3%, respectively. A multivariate analysis showed the ASA-PS (HR = 2.23, P = 0.026), serum Albumin ⩽ 4.0 g/dL (HR = 2.96, P = 0.007), T4 tumor (HR = 2.73, P = 0.002) and R1 resection (HR = 6.56, P = 0.02) to be independent risk factors for recurrence. Furthermore, poorly differentiated cancers (HR = 6.28, P = 0.009), a T4 tumor (HR = 3.46, P = 0.011) and R1 resection (HR = 6.16, P = 0.006) were independent prognostic factors in patients with obstructive CRC.

CONCLUSION: The outcomes of patients with obstructive CRC was poor. T4 tumor and R1 resection were found to be independent prognostic factors for both recurrence and survival in patients with obstructive CRC.

Key words: Obstructive colorectal cancer; Prognostic factor; Survival

Core tip: Obstructive colorectal cancer (CRC) still have poor prognosis. However, the prognostic factor of obstructive CRC is unclear. The aim of this article is to clarify the long-term outcome and the risk factors for obstructive CRC at multiple institutions. The five-year disease free survival and cancer-specific survival were 50.6% and 80.3%, respectively. T4 tumor and R1 resection were independent prognostic factors for both recurrence and survival.

Atsushi I, Mitsuyoshi O, Kazuya Y, Syuhei K, Noriyuki K, Masashi M, Akira W, Kentaro S, Nobuyuki K, Natsuko S, Jun W, Yasushi I, Chikara K, Itaru E. Long-term outcomes and prognostic factors of patients with obstructive colorectal cancer: A multicenter retrospective cohort study. *World J Gastroenterol* 2016; 22(22): 5237-5245 Available from: URL: http://www.

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in Japan, and the incidence of CRC has been increasing rapidly. CRC is difficult to diagnose due to it early atypical symptoms and signs. Around 7%-16% of patients with colorectal malignancy present with acute colorectal obstruction[1-3]. It is generally accepted that right sided obstructive CRC can best be treated by right hemicolectomy with ileocolic anastomosis. On the other hand, the optimal treatment for left-sided obstructive CRC remains controversial[4-7]. The treatment options range from an emergency radical operation, such as Hartmann's procedure, to bowel decompression using metallic stents or transanal tube or proximal diversion with a subsequent staged resection. The choices of surgical intervention for obstructive CRC vary greatly, according to the tumor location, general condition of the patients, and the experience level of the surgeons[8]. Therefore, It has been reported that CRC patients with obstruction have an advanced stage and worse long-term survival compared to non-obstructive CRC[3,9-11]. Although the negative impact of obstruction on the postoperative outcomes has been well documented, few studies have examined the outcomes of obstructive CRC patients in Japan[11-13]. Furthermore, the risk factors for recurrence and the prognostic factors are unclear owing to the small number of patients in previous study.

The aim of this study is to investigate the long-term oncologic outcomes and prognostic factors in patients with obstructive CRC at multiple Japanese institutions.

MATERIALS AND METHODS

Three hundred and sixty-two patients who were diagnosed to have obstructive colorectal cancer from January 2002 to December 2012 at the Yokohama Clinical Oncology Group's Department of Gastroenterological Surgery (10 institutions) were enrolled. Obstructive CRC was diagnosed based on medical history, physical examination, abdominal computed tomography (CT), and colonoscopy, and the surgical findings. We first performed emergency decompression of bowel obstruction by ileostomy/colostomy or the insertion of a decompression tube, or emergency resection of the primary lesion. The type of decompression method was chosen according to the surgeon's judgment and preference. Patients with distant metastatic lesions (n = 103), who only underwent stoma creation and best supportive care (n = 23), stage Ⅰ (n = 2) were excluded from this study. As a result, 234 patients who underwent surgical resection were analyzed retrospectively (Figure 1). The prognostic factors

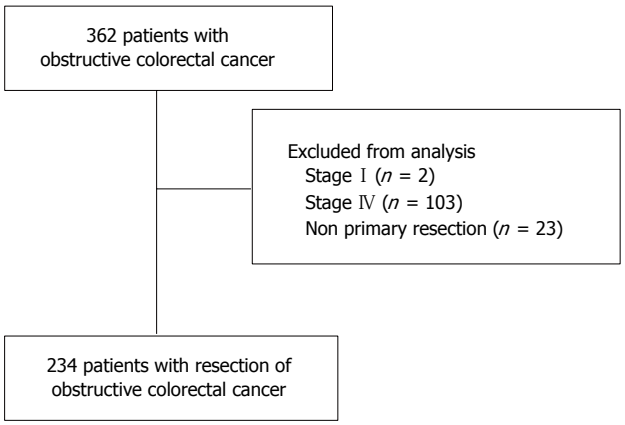

Figure 1 Study flowchart.

influencing survival and risk factors for recurrence were analyzed.

Clinicopathological information was obtained from the medical records of the patients including gender, age, The American Society of Anesthesiologists (ASA)-physical status (PS), serum albumin, CEA, preoperative decompression, location of the tumor, tumor size, differentiation of the tumor, depth of the tumor, intramural lymphatic invasion, intramural vascular invasion, lymph node dissection, number of lymph nodes harvested, lymph node involvement, postoperative complication, anastomotic leakage, curability, and adjuvant chemotherapy. There were missing values for BMI in 13 patients, for serum albumin in 14 patients, for CEA in 19 patients, for tumor size in 4 patients, for lymphatic invasion in one patient and for harvested lymph nodes in 10 patients because this was a retrospective study.

Japanese D3 lymphadenectomy is equivalent to complete mesocolic excision (CME) with central vascular ligation (CVL)[14]. D2 lymphadectomy includes pericolic and intermediate nodes region, and D0-1 includes only pericolic nodes region.

Statistical analysis

The disease-free survival (DFS) and cancer-specific survival (CSS) were estimated using the Kaplan-Meier method, and statistical significance was determined by the log-rank test. A multivariate analysis was performed using the Cox proportional hazard model to examine the independent prognostic factors and risk factors of recurrence. A *P* value of < 0.05 indicated statistical significance. All analyses were performed using the IBM SPSS, version 21 (SPSS Inc., Chicago, IL, United States).

This study received approval from the institutional review board of Yokohama City University.

RESULTS

Characteristic of the patients

The clinicopathological characteristics of the patients are summarized in Table 1. There were 234 patients who underwent surgical resection for obstructive CRC. The median age of the patients was 71 years (range 35-96) and there were 141 (60.3%) men and 93 (39.7%) women. Of these patients, 183 patients (72.2%) received preoperative decompression by colostomy/ileostomy (*n* = 56) or transanal tube insertion (*n* = 127)[15]. The most common tumor site was the sigmoid colon (*n* = 95). Other primary tumors were located in the descending colon (*n* = 36), ascending colon (*n* = 33), transverse colon (*n* = 33), rectum (*n* = 27), and cecum (*n* = 10). Among the 234 patients in this study, 165 patients (70.5%) had obstructing cancers at a site distal to the splenic flexure. T4 tumors were found in 125 patients (53.4%). There were 113 stage Ⅱ patients and 121 stage Ⅲ patients. In the stage Ⅲ cases, 90 patients had N1 disease and 31 patients had N2.

Table 1 Clinicopathological characteristic of patients with obstructive colorectal cancer *n* (%)

Variable	Category	*n* = 234
Gender	Male	141(60.3)
	Female	93 (39.7)
Age (yr)[1]		71 (35-96)
ASA	Ⅰ	70 (30)
	Ⅱ	128 (54.7)
	Ⅲ	36 (15.3)
Location of tumor	Cecum	10 (4.3)
	Ascending colon	33 (14.1)
	Transverse colon	33 (14.1)
	Descending colon	36 (15.4)
	Sigmoid colon	95 (40.6)
	Rectum	15 (10.5)
Decompression	+	183 (78.2)
	-	51 (21.8)
CEA (mg/dL)[1]		4.9 (0.3-2470)
Serum albumin[1]		3.4 (1.4-4.9)
Tumor size(mm)		48 (10-140)
Depth of tumor	pT3	109 (46.6)
	pT4a	93 (39.7)
	pT4b	32 (13.7)
Lymph node involvement	N0	113 (48.3)
	N1	90 (38.5)
	N2	31 (13.2)
R0 resection	+	219 (93.6)
	-	15 (6.4)
Adjuvant chemotherapy	+	91 (38.9)
	-	143 (61.1)

[1]Median (range).

A total of 219 patients (93.6%) underwent R0 resection of the primary lesion. The reasons of R1 resection (*n* = 15) were positive surgical margins in 11 patients, other organ involvement in 3 patients and residual lymph node metastasis in 1 patient.

Ninety-one of the 234 patients underwent adjuvant chemotherapy. The chemotherapeutic regimen was oral 5-fluorouracil (5-FU) in 47 patients, oral 5-FU plus leucovorin in 36, oxaliplatin-based chemotherapy in 5, and a Roswell Park Memorial Institute (RPMI) regimen in 3[16].

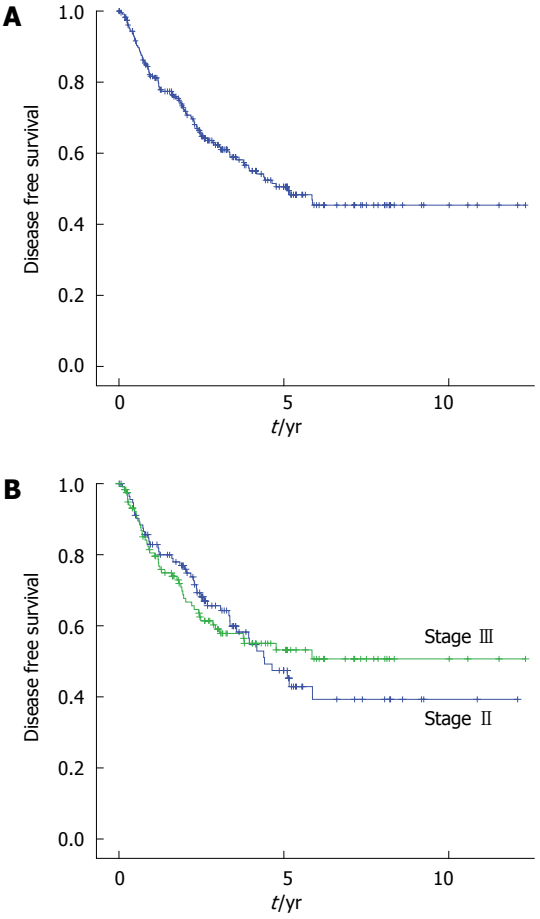

Figure 2 Kaplan-Meier curves showing the disease free survival after primary tumor resection in patients with obstructive colorectal cancer. A: All stage (n = 234); B: Stage Ⅱ (n = 114, blue line), Stage Ⅲ (n = 120, green line).

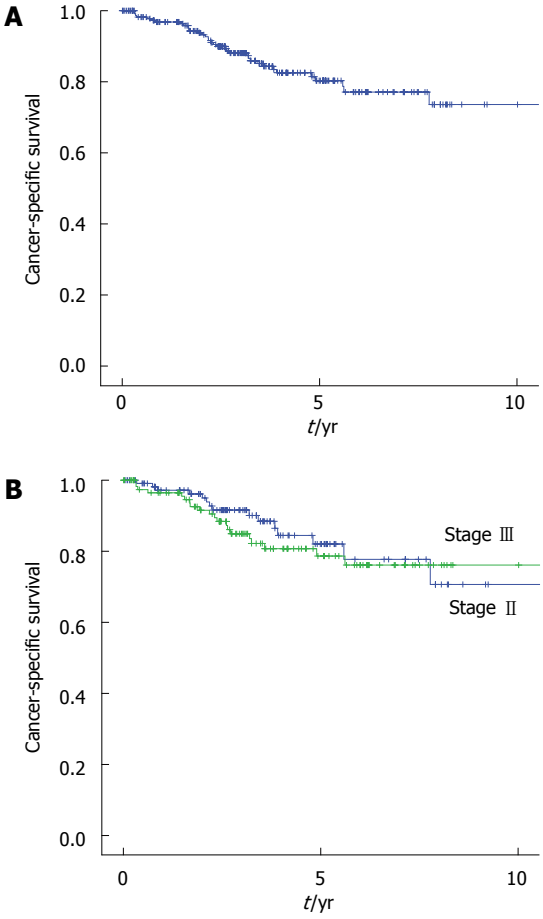

Figure 3 Kaplan-Meier curves showing the cancer-specific survival rates after primary tumor resection in patients with obstructive colorectal cancer. A: All stage (n = 234); B: Stage Ⅱ (n = 114, blue line), Stage Ⅲ (n = 120, green line).

Long-term outcomes

The median follow-up interval was 39 mo. The five-year DFS and CSS for all patients were 50.6% and 80.3%, respectively. The 5-year DFS of the patients with stage Ⅱ and stage Ⅲ disease were 47.4% and 55.1%, respectively. The 5-year CSS were 78.7% and 82.0%, respectively. There were no significantly differences in both the DFS and CSS between stage Ⅱ and stage Ⅲ disease (P = 0.856, P = 0.560) (Figures 2 and 3).

Recurrence site

A total 71 patients (30.3%) experienced recurrence during the study follow-up (Table 2). The most common site of recurrence was the liver (n = 27, 11.5%), followed by the lung (n = 22, 9.4%), peritoneum (n = 21, 9.0%), and local recurrence (n = 9, 3.8%). Other sites of recurrence included the non-regional lymph nodes (n = 6), anastomosis (n = 3), abdominal wall (n = 2), and pleural dissemination (n = 2). The rate of the recurrence sites did not differ substantially between stage Ⅱ and stage Ⅲ disease (data not shown).

Table 2 Patterns of recurrence following colorectal resection of obstructive colorectal cancer (n = 71)

Site of recurrence	n (%)
Liver	27 (11.5)
Lung	22 (9.4)
Peritoneal dissemination	21 (9.0)
Local recurrence	9 (3.8)
Lymph node	6 (2.6)
Anastomosis	3 (1.3)
Abdominal wall	2 (0.9)
Pleural dissemination	2 (0.9)

Risk factors for recurrence

The risk factors for recurrence according to our analysis are shown in Table 3. According to a univariate analysis, the factors associated with recurrence were age ⩾ 75 years (P = 0.011), ASA-PS (P = 0.017), serum albumin ⩽ 4.0 g/dL (P = 0.001), T4 tumor (P = 0.001), and R1 resection (P < 0.001). A multivariate analysis of these factors confirmed significant differences for ASA-PS (HR = 2.234, P = 0.026) serum albumin (HR = 2.967, P = 0.007), depth of tumor

Table 3 Result of the univariate and multivariate analysis of risk factors for recurrence

Factor		*n*	Univariate analysis			Multivariate analysis	
			3-yr RFS	5-yr RFS	*P* value	HR (95%CI)	*P* value
Gender	M	141	59.2%	47.6%	0.409		
	F	93	66.8%	54.7%			
Age (yr)	⩾ 75	96	55.0%	42.4%	0.011	1.228 (0.659-2.290)	0.518
	< 75	138	67.3%	55.9%			
ASA-PS	1	71	75.7%	64.8%	0.017	2.234 (1.101-4.535)	0.026
	2-3	163	56.6%	44.6%			
BMI (kg/m^2)	⩾ 25	28	66.0%	49.7%	0.951		
	< 25	193	62.7%	52.7%			
Serum albumin (g/dL)	⩽ 4.0	172	54.9%	41.3%	0.001	2.967 (1.342-6.560)	0.007
	> 4.0	48	79.7%	73.1%			
CEA (mg/dL)	⩾ 5.0	102	52.4%	44.8%	0.052		
	< 5.0	113	74.1%	54.2%			
Decompression	+	183	62.9%	50.7%	0.572		
	-	51	60.5%	50.4%			
Location of tumor	Right side	69	62.5%	55.5%	0.738		
	Left side	165	62.1%	48.7%			
Tumor size (mm)	⩾ 50	99	61.0%	48.8%	0.384		
	< 50	121	63.9%	54.1%			
Differentiation of tumor	tub1,tub2	222	63.7%	51.2%	0.080		
	por, muc	12	36.7%	36.7%			
Depth of tumor	T3	108	73.5%	64.7%	0.001	2.728 (1.467-5.072)	0.002
	T4	126	53.0%	38.7%			
Intramural lymphatic invasion	+	157	61.4%	53.2%	0.600		
	-	73	64.4%	45.2%			
Intramural vascular invasion	+	164	60.3%	49.3%	0.683		
	-	70	68.1%	57.4%			
Lymph node involvement	+	120	65.7%	47.4%	0.856		
	-	114	59.1%	53.2%			
Lymph node dissection	D0,D1	31	44.3%	44.3%	0.067		
	D2,D3	199	64.7%	50.8%			
No. of lymph nodes harvested	< 12	73	54.8%	44.5%	0.085		
	⩾ 12	151	66.4%	52.5%			
Postoperative complication	+	78	57.3%	44.7%	0.071		
(≥ Grade 2)	-	156	64.7%	53.5%			
Anastomotic leakage	+	18	71.1%	56.9%	0.562		
	-	191	61.7%	50.0%			
Curability	R0	219	66.4%	54.8%	< 0.001	6.555 (1.344-31.970)	0.020
	R1	15	7.6%	0.0%			
Adjuvant chemotherapy	+	91	66.2%	55.6%	0.069		
	-	143	59.8%	47.2%			

(HR = 2.728, *P* = 0.002) and curability (HR = 6.555, *P* = 0.02). There were no differences in the relapse rate according to whether the patients underwent preoperative decompression or not. Furthermore, lymph node involvement was also not associated with recurrence.

Prognostic factors for CSS

The prognostic factors for CSS are shown in Table 4. A univariate analysis identified age ⩾ 75 (*P* = 0.027), poorly or mucinous differentiation(*P* = 0.001), T4 tumor (*P* = 0.001), D0 or D1 lymph node dissection(*P* = 0.0014), R1 resection (*P* < 0.001) as poor prognostic factors. According to a multivariate analysis, poorly differentiated cancers or mucinous differentiation (HR = 6.282, *P* = 0.009), T4 tumor (HR = 3.458, *P* = 0.011) and R1 resection (HR = 6.162, *P* = 0.006) were independent prognostic factors in patients with obstructive CRC (Figure 4).

DISCUSSION

In the present study, we evaluated the long-term oncologic outcomes and prognostic factors in patients with obstructive CRC in multiple Japanese institutions. Most previous studies have reported that patients with obstructive CRC have significantly poorer oncologic outcomes than patients with nonobstructive CRC[9,11,12,17]. Obstructive tumors have been reported to have a more advanced stage than nonobstructive tumors[11,18]. The reported 5-year survival ranges between 36% to 64.6% in patients with obstructive CRC[11,17,19,20]. Our retrospective data showed 5-year CSS to be 80.3%, which was higher than the previously reported findings. One reason for the good outcomes might be that Japanese standard surgical procedures include complete tumor resection and extended D2/D3 lymph node dissection, including the pericolic, intermediate and most central lymph

Table 4 Result of the univariate and multivariate analysis of prognostic factors for cancer-specific survival

Factor		*n*	Univariate analysis			Multivariate analysis	
			3-yr CSS	5-yr CSS	*P* value	HR(95%CI)	*P* value
Gender	M	141	88.3%	80.8%	0.924		
	F	93	87.8%	79.7%			
Age (yr)	⩾ 75	96	82.2%	71.6%	0.027	1.464 (0.647-3.310)	0.360
	< 75	138	91.9%	85.4%			
ASA-PS	1	71	86.0%	83.5%	0.710		
	2-3	163	89.0%	78.7%			
BMI (kg/m^2)	⩾ 25	28	92.0%	85.8%	0.389		
	< 25	193	87.3%	80.0%			
Serum albumin (g/dL)	⩽ 4.0	172	85.6%	82.6%	0.536		
	> 4.0	48	87.7%	76.2%			
CEA (mg/dL)	⩾ 5.0	102	86.7%	80.0%	0.715		
	< 5.0	113	90.5%	82.0%			
Decompression	+	183	88.4%	81.7%	0.514		
	-	51	87.1%	74.8%			
Location of tumor	Right side	69	82.6%	70.5%	0.103		
	Left side	165	90.4%	84.3%			
Tumor size (mm)	⩾ 50	99	84.1%	76.9%	0.291		
	< 50	121	90.4%	85%			
Differentiation of tumor	tub1, tub2	222	89.9%	82.5%	0.001	6.282 (1.584-24.909)	0.009
	por, muc	12	50.0%	50.0%			
Depth of tumor	T3	108	95.7%	92.3%	0.001	3.458 (1.324-9.031)	0.011
	T4	126	81.1%	69.2%			
Intramural lymphatic invasion	+	157	90.8%	82.7%	0.449		
	-	73	86.9%	79.4%			
Intramural vascular invasion	+	164	94.2%	90.1%	0.152		
	-	70	85.9%	76.7%			
Lymph node involvement	+	120	92.5%	82.9%	0.332		
	-	114	84.0%	77.7%			
Lymph node dissection	D0, D1	31	60.7%	60.7%	0.014	0.958 (0.300-3.056)	0.942
	D2, D3	199	90.4%	83.0%			
No. of lymph nodes harvested	< 12	73	83.3%	78.3%	0.314		
	⩾ 12	151	91.1%	81.0%			
Postoperative complication	+	78	85.9%	75.6%	0.644		
(⩾ Grade 2)	-	156	89%	82.5%			
Anastomotic leakage	+	18	100%	100%	0.069		
	-	191	87.1%	78.7%			
Curability	R0	219	91.3%	84.7%	< 0.001	6.162 (1.692-22.445)	0.006
	R1	15	39.7%	19.9%			
Adjuvant chemotherapy	+	91	87.7%	81.8%	0.800		
	-	143	88.8%	79.4%			

nodes. West *et al* reported that the Japanese surgical procedures as well as CME with CVL eradicates tumors more effectively than the conventional procedures[14]. In our study, D2/D3 lymph node dissection was performed in about 70% of all patients.

Several authors have suggested preoperative obstruction to be a prognostic factor in CRC[12,17]. However, there are few data concerning the prognostic factors associated with obstructive CRC patients[4,19]. Jiang *et al*[4] reported a delayed resection to provide a better oncologic outcome than a primary resection for obstructive left-sided colorectal cancer. Other authors have showed that decompression followed surgery is better than emergency surgery in terms of the primary anastomosis rate, the stoma rate, the morbidity rate, the successful treatment of the patient's comorbidities, and preparation for elective surgery[21,22]. According to our results, however, no difference in the prognosis was found in regard to whether patients underwent preoperative decompression or not, It therefore remains inconclusive as to which approach may be superior to the other.

Malignant obstruction can occur in any part of the colon and rectum, however, the risk varies at different locations. In present study, 70.5% of the obstructive CRC occurred in the left-sided colon and most of them occurred in the sigmoid colon. This tumor distribution is similar to what has been reported by other series[11,19]. Our results showed that the prognosis was not different between right-sided and left-sided obstructive CRC.

Obstructive tumors are reported to be associated with a more advanced stage than nonobstruction[11,18]. Our data showed 53.4% to have T4 tumors, and 51.7% had positive lymph nodes. This is one of the reasons why obstructive CRC has a worse prognosis. In present study, especially, a T4 tumor was found to be a risk factor for recurrence in patients with

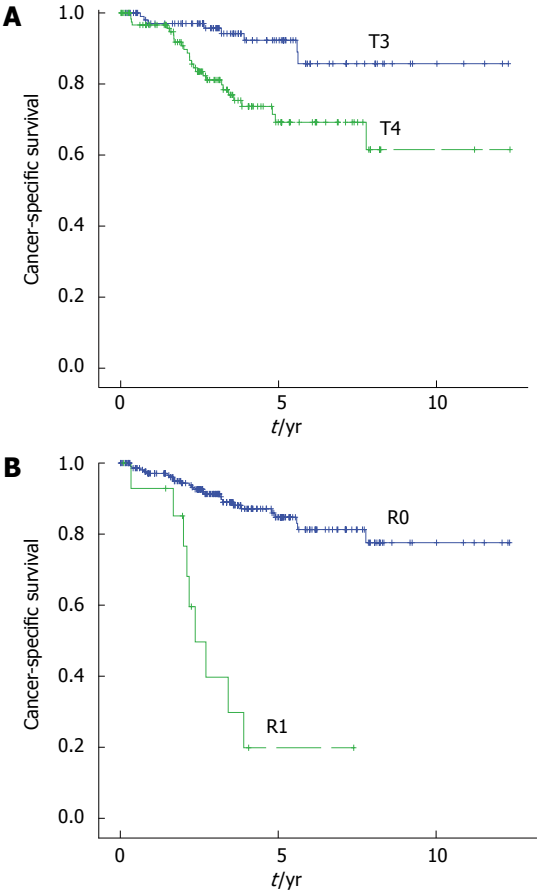

Figure 4 Kaplan-Meier curves showing the cancer-specific survival rates in patients with T4 tumor and R1 resection. A: T3 (*n* = 108, blue line); T4 (*n* = 126, green line); B: R0 (*n* = 219, blue line), R1 (*n* = 15, green line).

obstructive CRC.

Seventy-one patients had a recurrence of the disease as the first event in our study. The distant metastasis rate is significantly higher in obstructed patients when compared with nonobstructed patients[17]. The common sites of recurrence were the liver (11.5%) and lung (9.4%), which were similar to previous reports[23]. An interesting finding in the present study is that patients with obstructive CRC showed a higher rate of peritoneal dissemination (9.0%) than previously reported (1.9%-3.5%) in nonobstructive CRC[24,25]. These findings suggested that obstructive CRC was locally advanced cancer consisting of T4 tumors and which may be unexpectedly exposed during R1 resection. Therefore, reducing the rate of performing R1 resection might be a key to achieving improved surgical results.

In our study, patients with stage Ⅱ disease and those with stage Ⅲ disease had similar poor outcomes in terms of the 5-year DFS and CSS. This finding suggests that lymph node involvement, which is a well-known prognostic factor, does not have any significant impact on the outcomes in patients with obstructive CRC. One of the reasons for this finding is due to the fact that Japanese standard lymph node dissection procedures for advanced colorectal cancer include D2/D3 lymph node dissection, which is nearly the same method as that performed for CME and CVL[14]. In our clinical oncology group, lymphadenectomy for colorectal cancer was routinely performed during the study period. Therefore, a T4 tumor was identified as the most important prognostic factor, in which the 5-year DFS and OS were 38.7% and 60%, respectively.

In the present study, a lower level of albumin was also a predictive factor for survival. The serum albumin levels have recently been studied as the Glasgow Prognostic Score (GPS), based on a combination of albumin and C-reactive protein (CRP). Several authors have revealed the GPS to have prognostic value in patients with advanced colorectal cancer[26,27]. However, we failed to collect data of CRP because this was a retrospective analysis. It is estimated that low albumin levels are associated with a decreased survival time because a low albumin level likely reflects some type of systemic compromise[28].

Our results demonstrated that poorly differentiated tumors or mucinous differentiated tumors are also predictive factors for survival. Histologically, poorly differentiated CRC represents from 4.8% to 23.2% of all colorectal cancers[29]. The rate of poorly differentiated tumors was not higher than that described in previous reports. Poorly differentiated cancers have been linked to adverse prognoses in many studies[30].

Recently, several authors have suggested the feasibility of performing preoperative chemotherapy without the routine use of radiation therapy for locally advanced rectal cancer and a high R0 resection rate[31,32]. Furthermore, the FOxTROT Collaborative Group demonstrated the feasibility of performing preoperative chemotherapy for locally advanced colon cancer[33]. Our result suggested that obstructive colorectal cancer is also locally advanced cancer. Therefore, preoperative chemotherapy after the decompression of bowel obstruction may also be useful for the management of obstructive colorectal cancer.

Our retrospective study had several important limitations. First, there were several missing data and we could not obtain the clinical course related to the treatment of patients after recurrence. Second, the adjuvant therapy, which affects the outcome, was not uniform.

In conclusion, in addition to generally accepted knowledge, we found that T4 tumor and R1 resection were prognostic factors for both recurrence and survival. These results suggested that a curative resection of the tumor is very important and that systemic treatment for preventing distant metastasis, such as peritoneal dissemination associated with T4 tumors, is necessary in patients with obstructive colorectal cancer.

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COMMENTS

Background

Colorectal cancer (CRC) is one of most common cancer in the world. Around 7%-16% of patients with colorectal cancer present with acute colorectal obstruction. It has been reported that CRC patients with obstruction have an advanced stage and poor long-term survival compared to non-obstructive CRC. However, the risk factors for recurrence and the prognostic factors of patients with obstructive CRC are unclear.

Research frontiers

The authors often treat the obstructive CRC. However, there are few literatures concerning survival and prognostic factor of obstructive CRC. The research hotspot is to introduce long-term outcome of patients with obstructive colorectal cancer and prognostic factors in Japan.

Innovations and breakthroughs

The present study represents the characteristics and the long-term outcome of obstructive CRC patients in Japan and revealed that T4 tumor and R1 resection are risk factors of recurrence and prognostic factors. These results suggested that a curative resection of the tumor is very important and systemic treatment for preventing distant metastasis, such as peritoneal dissemination associated with T4 tumors, is necessary in patients with obstructive colorectal cancer.

Applications

This study showed the poor survival for obstructive colorectal cancer patients and prognostic factor. The present study provided readers the important information of treatment for patients with obstructive CRC.

Peer-review

The authors demonstrated that T4 tumor status and R1 resection are independent prognostic factors in patients with obstructive colorectal cancer.

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ORIGINAL ARTICLE

Retrospective Study

Post-discharge complications after esophagectomy account for high readmission rates

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Author contributions: Chen SY, Molena D, Stem M, Mungo B and Lidor AO designed the study, wrote the manuscript and made the decision to submit; Chen SY, Stem M and Lidor AO contributed to the data collection, analysis, interpretation.

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Data sharing statement: No additional data are available. American College of Surgeons National Surgical Quality Improvement Program and the hospitals participating in the ACS-NSQIP are the source of the data used herein; they have not verified and are not responsible for the statistical validity of the data analysis or the conclusions derived by the authors.

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Abstract

AIM: To identify rates of post-discharge complications (PDC), associated risk factors, and their influence on early hospital outcomes after esophagectomy.

METHODS: We used the 2005-2013 American College of Surgeons National Surgical Quality Improvement Program (ACS-NSQIP) database to identify patients ⩾ 18 years of age who underwent an esophagectomy. These procedures were categorized into four operative approaches: transhiatal, Ivor-Lewis, 3-holes, and non-gastric conduit. We selected patient data based on clinical relevance to patients undergoing esophagectomy and compared demographic and clinical characteristics. The primary outcome was PDC, and secondary outcomes were hospital readmission and reoperation. The patients were then divided in 3 groups: no complication (Group 1), only pre-discharge complication (Group 2), and PDC patients (Group 3). A modified Poisson regression analysis was used to identify risk factors associated with developing post-discharge complication, and risk ratios were estimated.

RESULTS: 4483 total patients were identified, with

8.9% developing PDC within 30-d after esophagectomy. Patients who experienced complications post-discharge had a median initial hospital length of stay (LOS) of 9 d; however, PDC occurred on average 14 d following surgery. Patients with PDC had greater rates of wound infection (41.0% *vs* 19.3%, $P < 0.001$), venous thromboembolism (16.3% *vs* 8.9%, $P < 0.001$), and organ space surgical site infection (17.1% *vs* 11.0%, $P = 0.001$) than patients with pre-discharge complication. The readmission rate in our entire population was 12.8%. PDC patients were overwhelmingly more likely to have a reoperation (39.5% *vs* 22.4%, $P < 0.001$) and readmission (66.9% *vs* 6.6%, $P < 0.001$). BMI 25-29.9 and BMI $\geqslant$ 30 were associated with increased risk of PDC compared to normal BMI (18.5-25).

CONCLUSION: PDC after esophagectomy account for significant number of reoperations and readmissions. Efforts should be directed towards optimizing patient's health pre-discharge, with possible prevention programs at discharge.

Key words: Reoperation; Hospital readmission; Post-discharge complications; Esophagectomy; Outcomes research

Core tip: In this study, we used the 2005-2013 ACS-NSQIP database to identify the rate of post-discharge complications, their associated risk factors, and their influence on early hospital readmission after esophagectomy. This report demonstrates that post-discharge complications after esophagectomy account for a significant number of reoperations and readmissions. We believe that implementing prevention strategies to decrease common post-discharge complications like venous thromboembolism and infection should be considered, and that directing our energies toward optimizing patient health prior to discharge may improve overall surgical outcomes.

Chen SY, Molena D, Stem M, Mungo B, Lidor AO. Post-discharge complications after esophagectomy account for high readmission rates. *World J Gastroenterol* 2016; 22(22): 5246-5253 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i22/5246.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i22.5246

INTRODUCTION

Esophagectomy is the mainstay treatment for localized esophageal carcinoma without medical contraindications[1]. It can also be indicated for certain benign conditions, including high-grade dysplasia in Barrett's esophagus, caustic ingestion, reflux esophagitis complications, esophageal stricture, and esophageal neuromotor dysfunction (achalasia, spasm, scleroderma)[2]. Over 5000 esophagectomies are performed in the United States and United Kingdom annually[3]. Despite improved surgical techniques and intensive care unit therapy[4], post-esophagectomy mortality and morbidity rates remain suboptimal, ranging from 7%-28%[5,6] and 10%-27%[7], respectively. Moreover, readmission rates after esophagectomy range from 5%-25%[8,9], and the overall 5-year survival rate following esophagectomy range from 15%-40%[10,11].

These dismal outcomes after esophagectomy, compounded with national health policy changes by the Centers for Medicare and Medicaid Services (CMS) and the Affordable Care Act that emphasize reducing hospital readmission rates to improve health care quality, have spurred growing interest in investigating quality measures related to esophagectomy. Although several studies have explored esophagectomy complications and readmission[12-16] none to date have delved into risk factors associated specifically with post-discharge complications (PDC) after esophagectomy. Given that approximately a third of post-operative surgical complications occur post-discharge, and that PDC may differ from pre-discharge complications[17], we hypothesize that several clinical factors may increase risk for developing PDC after esophagectomy.

Using 2005-2013 data from the American College of Surgeons National Surgical Quality Improvement Program (ACS-NSQIP), we sought to identify the rate of PDC, their associated risk factors, and their influence on early hospital outcomes including readmission and reoperation. We believe that understanding these risk factors will provide additional insights to improve esophagectomy quality outcomes.

MATERIALS AND METHODS

Data source

This study is a retrospective analysis using the 2005-2013 ACS-NSQIP database. ACS-NSQIP is a nationally-validated, risk-adjusted, and outcomes-based program created for the purpose of measuring and improving surgical quality care[18,19]. The program collects data on patients undergoing surgery from over 650 participating hospitals of varying size and academic affiliation[20]. Eligibility criteria for hospital participation include: hiring a surgical clinical reviewer who uses a standardized format to capture and review data from clinical records, identifying a Surgeon Champion to lead the program at the hospital, agreeing to program protocols, meeting minimum case standards, and paying an annual participation fee to ACS. Prospective, systematic data collection is performed on 150 preoperative and intraoperative variables, in addition to 30-d postoperative morbidity and mortality. This study was reviewed and approved by the Institutional Review Board of the Johns Hopkins University School of Medicine.

Study population

Patients ⩾ 18 years of age who underwent an esophagectomy (defined as current procedural terminology codes 43107, 43108, 43112, 43113, 43117, 43118, 43121, 43122, or 43123) were included. These procedures were then categorized into four operative approaches: transhiatal (if the chest was not entered and the stomach was used as a conduit), Ivor-Lewis (if the anastomosis was done in the chest and the stomach was used as a conduit), 3-holes (if the anastomosis was done in the neck, the chest was entered and the stomach was used as a conduit), and intestinal conduit (if any of the above approaches were used but intestine, either large or small, was used as a conduit). Patients who had missing data for days from operation to discharge, and days from operation to complication (for patients who had complication) were excluded. Patients who were not discharged within 30-d of operation were also excluded. Lastly, patients who died during initial hospitalization were excluded from the analysis that aimed to identify risk factors associated with PDC (univariate logistic regression), as these patients were not at risk for PDC.

Baseline characteristics of patients

We selected patient data based on clinical relevance to patients undergoing esophagectomy and compared demographic and clinical characteristics. Three groups of patients were defined: no complication (Group 1), only pre-discharge complication (Group 2), and PDC patients (Group 3). Patients with esophageal/gastric cancer were defined with a diagnosis (International Classification of Diseases, 9th Revision, codes of 150, 150.1, 150.2, 150.3, 150.4, 150.5, 150.8, 150.9, 151, 151.0).

Outcomes

The primary outcome was PDC, which we defined as an event for which the time interval (days) between the initial operation and a complication was greater than the interval from operation to discharge. The secondary outcomes included hospital readmission (2011-2013) and reoperation (2012-2013). Complication types included from ACS-NSQIP were investigated. Prolonged length of stay and prolonged operative time, defined as stay or time greater than the 75th percentile, respectively, were also investigated.

Statistical analysis

Categorical variables were compared using Pearson's χ^2 test or Fisher's exact test when appropriate. Student's *t*-test or ANOVA were used to compare continuous variables. Modified Poisson regression analysis was performed to identify factors associated with developing a PDC, and risk ratios (RR) were estimated. A *P*-value of $P < 0.05$ was determined statistically significant. We performed all data analyses and management using Stata/MP version 14 (StataCorp LP, College Station, TX, United States).

RESULTS

Study population

A total of 4872 patients underwent esophagectomy between 2005 and 2013. However, 389 (7.98%) patients were not discharged before the 30-day period and were excluded for this very reason. 4483 patients represented our study population, including 2497 (55.7%) patients who had no postoperative complications, 1588 (35.4%) who had at least one pre-discharge complication, and 398 (8.9%) who had at least one PDC. The mean age was 63.1 years, with 80.0% male and 85.9% whites. The mean BMI was 27.9 kg/m^2. PDC patients tended to be slightly older with greater ASA class, BMI, and more comorbidities including diabetes and, dyspnea compared to no complication group (Table 1).

Unadjusted outcomes

The overall PDC rate was 8.9%. Patients who experienced PDC had a median initial length of hospital stay of 9 d; however, PDC occurred on average 14 d after surgery. Among procedure types, PDC rates were 35.7% for transhiatal, 34.1% for Ivor Lewis, 36.9% for 3-holes, and 44.3% for intestinal conduit. Interestingly, PDC rates remained similar over the studied years (8.3% in 2005-2006 *vs* 8.9% in 2013) (Figure 1). Of the patients who experienced PDC, 127 of them (31.9%) also had pre-discharge complications. The overall readmission rate (2011-2013) after esophagectomy was 12.8%. Only from 2012 NSQIP identified if readmissions were likely related to the principle surgical procedure. There were 253 readmissions (253/1989, 12.72%) between 2012 and 2013 and 83.8% (212/253) of these readmissions were related to the initial surgical procedure. PDC patients were overwhelmingly more likely to have a reoperation (39.5% *vs* 22.4% for 2012-2013, $P < 0.001$) and to be readmitted (66.9% *vs* 6.6% for 2011-2013, $P < 0.001$). Moreover, pre-discharge and PDC differed by complication types (Figure 2). PDC patients had greater rates of wound infection (41.0% *vs* 19.3%), VTE (16.3% *vs* 8.9%), and organ space SSI (17.1% *vs* 11.0%) ($P \leqslant 0.001$ for each) than pre-discharge complication patients (Table 2). Although 30-day mortality rates were greater for patients who experienced PDC, this finding was not statistically significant.

Risk factors associated with post-discharge complications

Univariate modified Poisson regression analysis revealed that greater BMI, specifically BMI 25-29.9 (RR = 1.37, 95%CI: 1.08-1.74) and BMI ⩾ 30 (RR = 1.34, 95%CI: 1.04-1.72), were associated with increased risk of PDC, compared to normal BMI (18.5-25) (Table 3).

Table 1 Baseline demographic and clinical characteristics of patients undergoing esophagectomy *n* (%)

Characteristic	Group 1 No complication *n* = 2497 (55.70)	Group 2 Pre-discharge complication *n* = 1588 (35.42)	Group 3 Post-discharge complication *n* = 398 (8.88)	*P* value
Age, mean (median)	62.5 ± 11.2 (63)	63.9 ± 10.8 (65)	63.4 ± 10.9 (64)	0.001
Age group (yr)				0.048
< 60	921 (36.88)	513 (32.30)	143 (35.93)	
60-69	862 (34.52)	579 (36.46)	126 (31.66)	
70-79	602 (24.11)	407 (25.63)	109 (27.39)	
⩾ 80	112 (4.49)	89 (5.60)	20 (5.03)	
Male[1]	2042 (81.88)	1220 (76.83)	325 (81.66)	< 0.001
Race				0.088
White	2135 (85.50)	1363 (85.83)	352 (88.44)	
Black	76 (3.04)	62 (3.90)	6 (1.51)	
Other/unknown	286 (11.45)	168 (10.26)	40 (10.05)	
ASA classification[1]				< 0.001
No disturb/mild disturb	611 (24.50)	237 (14.92)	88 (22.17)	
Serious disturb	1746 (70.01)	1161 (73.11)	269 (67.76)	
Life threat/moribund	137 (5.49)	190 (11.96)	40 (10.08)	
Body mass index, mean (median)	27.9 ± 6.4 (27)	27.7 ± 6.3 (26.9)	28.4 ± 6.2 (27.6)	0.132
Body mass index group[1] (kg/m^2)				0.013
< 18.5	68 (2.75)	59 (3.75)	9 (2.26)	
18.5-24.9	761 (30.72)	522 (33.14)	100 (25.13)	
25-29.9	905 (36.54)	532 (33.78)	159 (39.95)	
⩾ 30	743 (30.00)	462 (29.33)	130 (32.66)	
Diabetes	341 (13.66)	300 (18.89)	75 (18.84)	< 0.001
Current smoker	616 (24.67)	434 (27.33)	86 (21.61)	0.033
Dyspnea	209 (8.37)	205 (12.91)	48 (12.06)	< 0.001
History of COPD	131 (5.25)	156 (9.82)	23 (5.78)	< 0.001
Weight loss	468 (18.74)	301 (18.95)	73 (18.34)	0.959
Steroid use	74 (2.96)	42 (2.64)	12 (3.02)	0.820
Emergency case	18 (0.72)	39 (2.46)	3 (0.75)	< 0.001
Diagnosis				0.002
Benign disease	355 (14.22)	289 (18.20)	56 (14.07)	
Esophageal/gastric cancer	2142 (85.78)	1299 (81.80)	342 (85.93)	
Year of operation				0.012
2005-2007	293 (11.73)	223 (14.04)	41 (10.30)	
2008-2010	746 (29.88)	409 (25.76)	109 (27.39)	
2011-2013	1458 (58.39)	956 (60.20)	248 (62.31)	

[1]Different denominator due to missing data: gender (Total *n* = 4480; Group 1 *n* = 2494; Group 2 *n* = 1588; Group 3 *n* = 398); functional status (*n* = 4481; 2497; 1586; 398); ASA class (*n* = 4479; 2494; 1588; 397); BMI (*n* = 4450; 2477; 1575; 398). ASA: American Society of Anesthesiology; COPD: Chronic obstructive pulmonary disease; BMI: Body mass index.

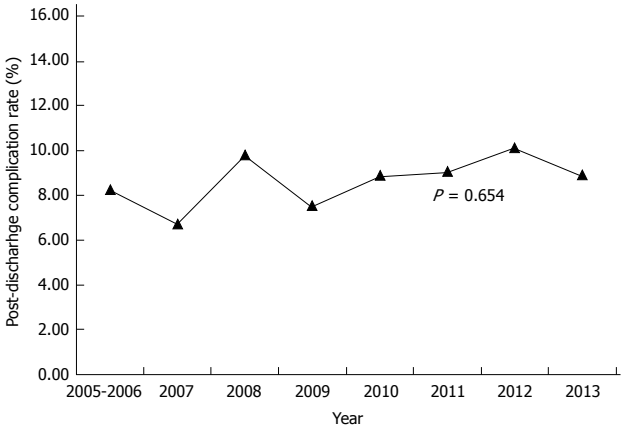

Figure 1 Post-discharge complications rates after esophagectomy by year.

DISCUSSION

Although several studies have investigated post-operative complications and readmissions following esophagectomy, none to our knowledge have explored the distinct role of PDC on early hospital outcomes. This is the first study to use ACS-NSQIP to examine the rate of PDC, their associated risk factors, and their influence on early hospital outcomes after esophagectomy. ACS-NSQIP offers a unique opportunity to assess at a national, multi-institutional level these specific health quality measures that may be unavailable in other large, population-level databases. Our retrospective analysis demonstrates that PDC occur at a low rate but account for a significant number of reoperations and readmissions.

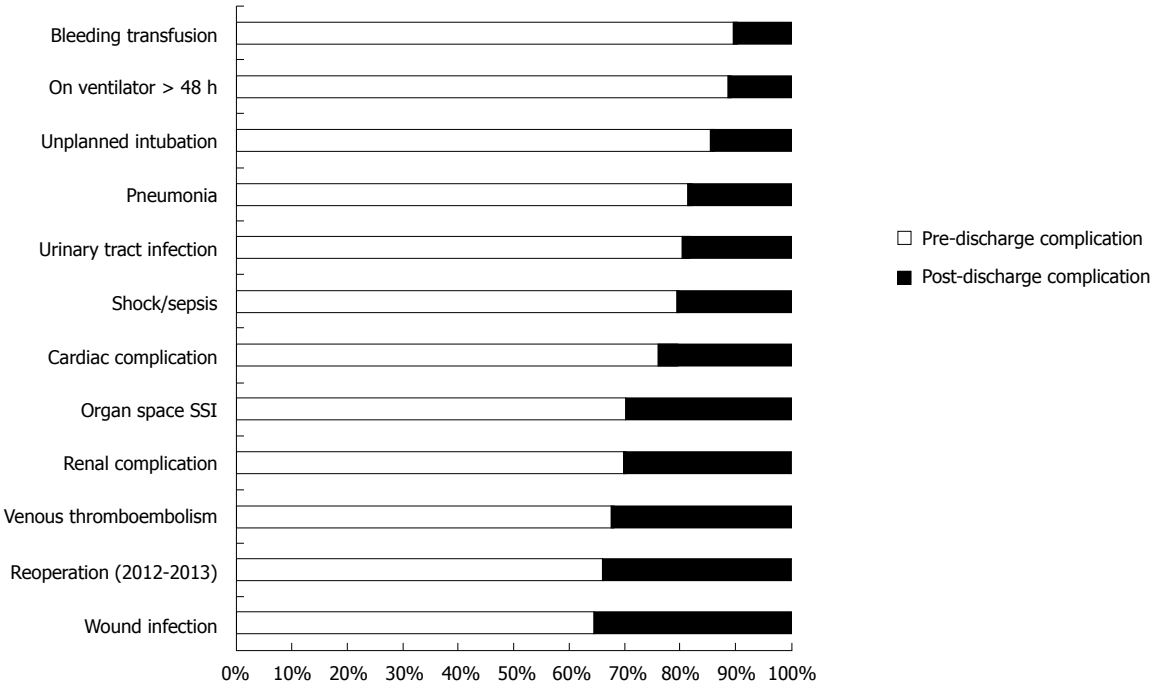

Figure 2 Proportion of pre-discharge *vs* post-discharge complications after esophagectomy for each morbidity.

Table 2 Observed unadjusted rates of pre- and post-discharge outcomes after esophagectomy *n* (%)

Outcome	Total (*n* = 1986)	Group 2 Pre-discharge complication *n* = 1588 (79.96)	Group 3 Post-discharge complication *n* = 398 (20.04)	*P* value
30-d mortality[1]	140 (7.05)	104 (6.55)	36 (9.05)	0.082
Overall morbidity				
Wound infection	470 (23.67)	307 (19.33)	163 (40.95)	< 0.001
Pneumonia	535 (26.94)	444 (27.96)	91 (22.86)	0.040
Urinary tract infection	112 (5.64)	91 (5.73)	21 (5.28)	0.725
Venous thromboembolism	207 (10.42)	142 (8.94)	65 (16.33)	< 0.001
Cardiac complication	108 (5.44)	92 (5.79)	16 (4.02)	0.163
Shock/sepsis	518 (26.08)	422 (26.57)	96 (24.12)	0.319
Unplanned intubation	420 (21.15)	368 (23.17)	52 (13.07)	< 0.001
Bleeding transfusion	584 (29.41)	526 (33.12)	58 (14.57)	< 0.001
Renal complication	59 (2.97)	50 (3.15)	9 (2.26)	0.351
On ventilator > 48 h	439 (22.10)	396 (24.94)	43 (10.80)	< 0.001
Organ space SSI	242 (12.19)	174 (10.96)	68 (17.09)	0.001
Reoperation 12-13	235 (25.97)	160 (22.38)	75 (39.47)	< 0.001
Serious Morbidity[2]	1102 (55.49)	901 (56.74)	201 (50.50)	0.025
Year of operation				0.142
2005-2007	264 (13.29)	223 (14.04)	41 (10.30)	
2008-2010	518 (26.08)	409 (25.76)	109 (27.39)	
2011-2013	1204 (60.62)	956 (60.20)	248 (62.31)	
Length of stay, d (median)	11.7 ± 5.4 (10)	15.0 ± 6.3 (14)	10.3 ± 4.3 (9)	< 0.001
Prolonged length of stay[3]	765 (38.52)	707 (44.52)	58 (14.57)	< 0.001
Operative time, min (median)	342.3 ± 134.7 (328)	358.6 ± 145.6 (343)	350.4 ± 133.1 (335.5)	0.308
Prolonged operative time[4]	570 (28.70)	467 (29.41)	103 (25.88)	0.164
Readmission 2011-2013	226 (19.07)	62 (6.60)	164 (66.94)	< 0.001

[1]In-hospital and post-discharge mortality among patients with pre or post-discharge complications. The overall mortality rate was 3.12% (140/4483); [2]Serious morbidity: cardiac complication, shock/sepsis, unplanned intubation, on ventilator > 48 h, organ space SSI, and reoperation 2012-2013; [3]Defined as length of stay > 75th percentile; [4]Defined as operative time > 75th percentile. SSI: Surgical site infection.

Higher BMIs were associated with increased risk of PDC. PDC were different than pre-discharge complications and potentially preventable.

Of the patients with PDC, wound infection, pneumonia, and VTE were among common PDC that could serve as targeted areas for quality improvement. These PDC are consistent with prior studies examining perioperative complications after esophagectomy[21,22].

Table 3 Unadjusted risk and risk ratio for post-discharge complications after esophagectomy

Risk factor	PDC risk (*n*/total)	RR (95%CI)
Overall PDC risk	398/4379 (9.09)	-
Procedure type		
Ivor-Lewis	196/2219 (8.83)	Ref
Transhiatal	115/1261 (9.12)	1.03 (0.83-1.29)
3-holes	66/730 (9.04)	1.02 (0.78-1.34)
Intestinal conduit	21/169 (12.43)	1.41 (0.92-2.15)
Age group (%)		
< 60	143/1562 (9.15)	Ref
60-69	126/1529 (8.24)	0.90 (0.72-1.13)
70-79	109/1082 (10.07)	1.10 (0.87-1.39)
⩾ 80	20/206 (9.71)	1.06 (0.68-1.65)
Male (%)	325/3505 (9.27)	1.11 (0.87-1.41)
Race (%)		
White	352/3763 (9.35)	Ref
Black	6/137 (4.38)	0.46 (0.21-1.00)
Other/unknown	40/479 (8.35)	0.89 (0.65-1.22)
ASA classification (%)		
No disturb/mild disturb	88/930 (9.46)	Ref
Serious disturb	269/3104 (8.67)	0.92 (0.73-1.15)
Life threat/moribund	40/341 (11.73)	1.24 (0.87-1.76)
Body mass index (%)		
18.5-24.9	100/1347 (7.42)	Ref
< 18.5	9/129 (6.98)	0.94 (0.49-1.81)
25-29.9	159/1561 (10.19)	1.37 (1.08-1.74)
⩾ 30	130/1310 (9.92)	1.34 (1.04-1.72)
Diabetes (%)	75/692 (10.84)	1.24 (0.98-1.57)
Current smoker (%)	86/1111 (7.74)	0.81 (0.64-1.02)
Dyspnea (%)	48/436 (11.01)	1.24 (0.93-1.65)
History of COPD (%)	23/297 (7.74)	0.84 (0.56-1.26)
Weight loss (%)	73/812 (8.99)	0.99 (0.77-1.26)
Steroid use (%)	12/124 (9.68)	1.07 (0.62-1.84)
Emergency case	3/52 (5.77)	0.63 (0.21-1.90)
Esophageal/gastric cancer (%)	342/3707 (9.23)	1.11 (0.84-1.45)
Prolonged length of stay[1] (%)	58/938 (6.18)	0.63 (0.48-0.82)
Prolonged operative time[2] (%)	103/1087 (9.48)	1.06 (0.85-1.31)

[1]Defined as length of stay > 75th percentile; [2]Defined as operative time > 75th percentile. PDC: Post-discharge complication; RR: Risk ratio; ASA: American Society of Anesthesiology; COPD: Chronic obstructive pulmonary disease.

Interventions to reduce PDC, such as adopting best practices to prevent wound infection and VTE, may improve esophagectomy outcomes. In one study, selective anticoagulant thromboprophylaxis using a DVT risk factor index significantly decreased DVT rates after esophageal cancer surgery[23]. Another study showed that low molecular weight heparin prophylaxis resulted in a 72% reduction in DVT risk after general surgery[24]. These suggest that efforts aimed to reduce common complications after surgery may also be effective and applicable to esophagectomy and should be continued after discharge. Interestingly, PDC rates have remained similar over the studied years, revealing likely no significant changes in practice or indications.

Our modified Poisson regression analysis demonstrates several factors that may increase the risk of PDC and therefore enable us to stratify higher-risk patients for perioperative risk assessment. These include patients with BMI 25.0-29.9 and BMI ⩾ 30, compared to normal BMI 18.5-25.0. Although obesity is associated with higher incidence of medical comorbidities like hypertension, diabetes, and cardiovascular disease[25], the association of BMI and complications after esophagectomy is conflicting. Several studies have suggested that overweight and obese patients may have increased risk for complications after esophagectomy, such as longer operative times[26] and greater risks for anastomotic leaks[27], respiratory complications[27], and surgical site infections[28]. However, other studies have shown that obese patients do not have more postoperative complications and longer hospital stays compared to non-obese patients[14,29,30]. Several routine measures implemented in the hospital setting to decrease the most common post-operative complications (*i.e.,* early mobilization, incentive spirometry and pulmonary toilet, DVT prophylaxis, daily dressing changes) are not continued after discharge, and the lack of preventive actions may impact obese patients more than non-obese. As such, appropriate interventions for higher BMI patients may be beneficial and may include enhanced perioperative management of patient comorbidities, preoperative risk stratification, additional patient education, continuation of pulmonary toilet, exercise programs, and DVT prophylaxis after discharge and earlier follow-up. For example, one study has shown that performing preoperative risk analysis on pulmonary function and general status when selecting patients for transthoracic esophagectomy reduced postoperative morbidity rates[31]. Interventions targeting higher BMI patients undergoing esophagectomy may therefore reduce PDC rates.

We decided to separate the patients with pre-discharge complications to keep our groups as homogeneous as possible for fair comparison. Since these patients have a longer hospital stay (median LOS 14 d), a 30-d follow-up may not be long enough to record PDC. It was interesting, however, to see that the most common types of PDC are different than those occurring in the initial post-operative period. This information is helpful to the physician to identify patients at risk of PDC and to plan preventative measures.

The overall readmission rate in our study is 12.8%, consistent with another study's rate of 18.6%[12]. From our study, 39.5% of PDC patients underwent reoperation (2012-2013) compared to 22.4% of pre-discharge complication patients. This suggests that reoperation may have been indicated due to complications that developed after discharge and subsequent delays in management. Even more striking is our finding that 66.9% of PDC patients were readmitted, compared to 6.6% of pre-discharge complication patients. Recent changes in health policy and reimbursements have brought issues of health care costs to the forefront, resulting in some hospital

administrations advocating for earlier discharge of patients. Readmission has become a major focus from health care quality and cost-savings standpoints. However, the use of readmission as a quality metric is still debatable[32,33]. In the case of esophagectomy, efforts to reduce costs by promoting earlier discharge may increase readmission[16], reoperation, and PDC rates; hospital readmission after esophagectomy for esophageal cancer is also associated with poor survival[12]. One study has shown that postoperative complications lead to greater readmission rates after colon resection[34]. In bariatric surgery patients, PDC account for a substantial source of patient morbidity and readmissions[17]. Rather than emphasizing shorter hospital lengths of stay, putting more energy into preventing complications after esophagectomy, and optimizing patient health prior to discharge instead may lead to improved surgical quality outcomes and reduced hospital spending.

Despite the advantages of ACS-NSQIP, the database does pose some limitations to our study. ACS-NSQIP does not contain consistent information on tumor histology, margins, stage, surgical history, neoadjuvant chemotherapy (within 30 d preoperatively), and neoadjuvant radiation (within the last 90 d) that could otherwise provide greater context in interpreting our results. Several complications common to esophagectomy, such as anastomotic leaks, chyle leak, and delayed gastric emptying, are not captured in the database. ACS-NSQIP also identifies 30-d postoperative readmission rather than 30-d post-discharge readmissions, which may introduce immortal person-time bias and lead to shorter number of follow-up days for patients with longer hospital stays. Although PDC could lead to greater mortality, because ACS-NSQIP does not capture data beyond 30-d postoperatively, further studies examining long-term outcomes including mortality is warranted. Readmission data is available only from 2011-2013, but the large patient sample population within that cohort should prevent significant alterations in our conclusions. It is also uncertain whether our findings are applicable to all hospital settings, as hospital participation is voluntary and comprised primarily of academic, tertiary-care centers. Hospital size, setting, patient volume, teaching status, and individual surgeon experience also cannot be adjusted for.

In summary, PDC after esophagectomy account for a significant number of reoperations and readmissions. Adopting best practices to reduce common PDC like VTE and infection, and performing interventions for higher-risk individuals such as those with high BMI, should therefore be considered.

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COMMENTS

Background

Esophagectomy is the mainstay treatment for esophageal carcinoma and can also be indicated for benign conditions with end-stage organ dysfunction or perforation. Despite improved surgical and medical care, post-esophagectomy mortality and morbidity rates remain suboptimal. Such disappointing outcomes after esophagectomy, compounded with national health policy changes that emphasize reducing hospital readmission rates and improving health care quality, have spurred growing interest in investigating quality measures related to esophagectomy. Using 2005-2013 data from the American College of Surgeons National Surgical Quality Improvement Program (ACS-NSQIP), we sought to identify the rate of post-discharge complications, their associated risk factors, and their influence on early hospital outcomes including readmission and reoperation.

Research frontiers

Although several studies have explored post-esophagectomy complications and readmission, none to date have delved into specifics of post-discharge complications and risk factors associated with them. The results from this study offer additional insights to improve esophagectomy quality outcomes.

Innovations and breakthroughs

No other studies have investigated the specific outcomes of post-discharge complications after esophagectomy. In this study, The authors demonstrated that post-discharge complications after esophagectomy occur a median of 14 d postoperatively and account for a significant number of reoperations and readmissions. Moreover, pre- and post-discharge complications differed by type, with venous thromboembolism and infection occurring more commonly after discharge.

Applications

These research findings can be applied to predict, identify, and prevent adverse outcomes in patients who have undergone esophagectomies. Implementing strategies to decrease common post-discharge complications like venous thromboembolism and infection should be considered, and directing our energies toward optimizing patient health prior to discharge may improve overall surgical outcomes.

Peer-review

This is a review of the ACS-NSQIP esophagectomy database, meant to identify post-discharge complications. It has a huge sample, a clear presentation and analysis, and an interesting discussion. It is without doubt an important topic deserving of evaluation.

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ORIGINAL ARTICLE

Retrospective Study

Clinical significance of HOTAIR expression in colon cancer

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Abstract

AIM: To detect the expression of the long noncoding RNA HOTAIR in colon cancer and analyze its relationship with clinicopathological parameters of colon cancer.

METHODS: Total RNA was extracted from 80 colon cancer tissues and matched tumor-adjacent normal colon tissues and reverse transcribed. Quantitative polymerase chain reaction was used to detect the expression of HOTAIR. The relationship between the expression of HOTAIR and clinicopathological parameters of colon cancer was analyzed.

RESULTS: The expression of HOTAIR was significantly higher in colon cancer tissues than in matched tumor-adjacent normal colon tissues ($P < 0.05$). HOTAIR expression was significantly higher in cases with lymph node metastasis than in those without metastasis; in lowly differentiated and undifferentiated cases than in highly and moderately differentiated cases; and in stages Ⅲ + Ⅳ cases than in stages Ⅰ + Ⅱ cases ($P < 0.05$).

CONCLUSION: HOTAIR expression is upregulated in colon cancer, suggesting that HOTAIR plays an important role in the tumorigenesis, development and metastasis of colon cancer. HOTAIR may act as an oncogene and represents a new molecular target for the treatment of colon cancer.

Key words: HOTAIR; Long non-coding RNA; Oncogene; Colon tumor

Core tip: This study aimed to detect the expression of HOTAIR in colon cancer and analyze its relationship with clinicopathological parameters of colon cancer. Total RNA was extracted from 80 colon cancer tissues and matched tumor-adjacent normal colon tissues and reverse transcribed. HOTAIR expression was upregulated in colon cancer, suggesting that it may play an important role in the tumorigenesis, development and metastasis of colon cancer. HOTAIR might acts as an oncogene and could be a new molecular target for the treatment of colon cancer.

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INTRODUCTION

Colon cancer is a clinically common, highly malignant tumor of the digestive tract. Although drugs targeting epidermal growth factor receptor (EGFR) and KRAS mutations have significantly extended the survival of some colon cancer patients[1-3], only a small number of patients can benefit from these drugs because of the complex etiology of this malignancy. Overall, the effects of current therapies for colon cancer are not satisfactory[4,5].

Long noncoding RNAs (lncRNAs) are non-protein coding transcripts of around 200 nucleotides, which exist widely in the genome and can regulate gene expression[6]. HOTAIR is one of the extensively studied lncRNAs in recent years. Many studies have indicated that HOTAIR plays an important role in breast cancer, pancreatic cancer, liver cancer, gastric cancer, esophageal cancer and non-small cell lung cancer[7-10]. Studies in colon cancer suggest that HOTAIR is an important oncogene that affects the biological behavior of colon cancer[11] and can serve as an independent risk factor[12]. The latest research suggests that the expression of HOTAIR is associated with tumor metastasis[13].

In the present study, we detected the expression of HOTAIR in 80 colon cancer tissue samples by quantitative polymerase chain reaction (qPCR). Based on the clinical and pathological parameters of colon cancer patients, we analyzed the possible role of HOTAIR in colon cancer development, metastasis and sensitivity to treatment, with an emphasis on the role of HOTAIR in colon cancer treatment. The findings will provide a theoretical basis for developing a new, targeted therapy for colon cancer.

MATERIALS AND METHODS

Clinical materials and reagents

Eighty patients with pathologically proven colon cancer who underwent surgery at our hospital from September 2011 to September 2013, and had complete clinical records, were included. All patients provided written informed consent, and the study protocol was approved by the Medical Ethics Committee of Zhengzhou University. The mean age of the patients was 64 ± 16 years. There were 46 patients with stage Ⅰ or Ⅱ disease, and 34 patients with stage Ⅲ or Ⅳ disease. Forty-one patients had well or moderately differentiated tumors, and 34 patients had poorly differentiated or undifferentiated tumors. No patients had undergone radiotherapy or chemotherapy before surgery. Tumor tissues and normal colon tissues at least 7 cm away from the tumor were taken, frozen in liquid nitrogen within 30 min and preserved for further use.

Trizol was purchased from Invitrogen. The reverse transcription kit and DNA ladder were purchased from Takara. Primers for qPCR were designed and synthesized by Shanghai GenePharma. The qPCR kit was purchased from Thermo.

RNA preparation and reverse transcription

Tissue samples preserved in liquid nitrogen were put into an RNase-free mortar with liquid nitrogen and pulverized. For each 100 mg of tissue, 1 mL of Trizol was added. RNA preparation was then performed following the manufacturer's instructions. The obtained RNA was dissolved in DEPC-treated water, and the RNA concentration was measured using a micro UV-Vis fluorescence spectrophotometer (e-spect, Malcom, Japan). The obtained RNA was preserved at -80 ℃ for further use.

RNA reverse transcription was performed using a reverse transcription kit in a 20-μL system, containing 11 μL of DEPC-treated water, 1 μL of total RNA, 4 μL of 5 × buffer, 1 μL of RNase inhibitor, 2 μL of dNTPs, and 1 μL of reverse transcriptase. Reaction parameters were 42 ℃ for 60 min and 95 ℃ for 5 min. The obtained cDNA was preserved at -80 ℃ for further use.

qPCR: qPCR was performed in a 20-μL system containing 1 μL of cDNA, 10 μL of 2 × Master Mix with 0.03 × ROX added, 1 μL of forward primer (final concentration of 0.5 μmol/L), 1 μL of reverse primer, and 8 μL of DEPC-treated water on a Mx3005p cycler. PCR amplification was performed in triplicate. Cycling parameters were 95 ℃ for 7 min, followed by 40 cycles of 95 ℃ for 15 s and 60 ℃ for 30 s.

Statistical analysis

The expression levels of HOTAIR in tissues are expressed as mean ± SD and were compared using a

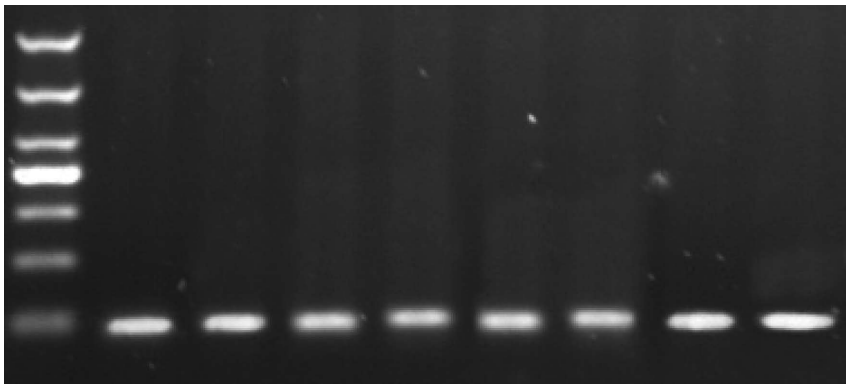

Figure 1 Quantitative polymerase chain reaction products of HOTAIR.

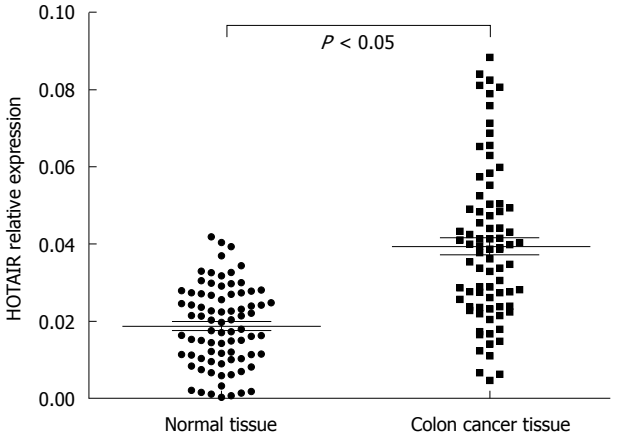

Figure 2 Expression of HOTAIR in colon cancer. The expression of HOTAIR in colon cancer is significantly higher in colon cancer tissues than in tumor-adjacent normal colonic tissues.

two-sample *t*-test. Statistical analyses were performed using SPSS13.0. *P*-values < 0.05 were considered statistically significant.

RESULTS

Agarose gel electrophoresis of qPCR products

The length of the expected PCR product for HOTAIR is 91 bp, and agarose gel electrophoresis showed that qPCR yielded PCR products of expected size (Figure 1).

Expression of HOTAIR is higher in colon cancer tissues than in tumor-adjacent normal colonic tissues

QPCR analysis showed that, although the expression of GAPDH showed no significant differences, the Ct value of HOTAIR was significantly lower in colon cancer tissues than in tumor-adjacent normal colonic tissues, suggesting that HOTAIR expression is upregulated in colon cancer. When the relative expression level is expressed as N ($N = 2^{-\Delta Ct}$, $\Delta Ct = Ct_{HOTAIR} - Ct_{GAPDH}$[14]), the relative expression level of HOTAIR was significantly higher in colon cancer tissues than in tumor-adjacent normal colonic tissues ($P < 0.05$, Figure 2).

Relationship between HOTAIR expression and clinicopathological parameters in colon cancer

HOTAIR expression was significantly correlated with lymph node metastasis, tumor differentiation and TNM stage ($P < 0.05$). HOTAIR expression was significantly higher in cases with lymph node metastasis than in those without metastasis, in lowly differentiated and undifferentiated cases than in highly and moderately differentiated cases, and in stages Ⅲ + Ⅳ cases than in stages Ⅰ + Ⅱ cases. By contrast, HOTAIR expression had no significant correlation with patient gender, age or tumor size ($P > 0.05$) (Tables 1-3).

Relationship between HOTAIR expression and survival in colon cancer

The Kaplan-Meier method was used to assess the impact of HOTAIR expression on survival of patients with colon cancer. The cumulative survival rate was significantly higher in patients with low HOTAIR expression than in those with high HOTAIR expression ($P < 0.05$) (Figure 3).

Risk factors for prognosis of colon cancer patients

Using prognosis of colon cancer patients as the dependent variable and factors possibly influencing the prognosis as independent variables, Cox multiple regression analysis was performed. The results showed that TNM stage, lymph node metastasis and HOTAIR expression were independent risk factors for prognosis of colon cancer patients.

Relationship between HOTAIR expression and prognosis in colon cancer

The relationship between prognosis of colon cancer patients after chemotherapy and HOTAIR expression was analyzed. The results showed that high HOTAIR expression was associated with poorer prognosis (Figure 4).

DISCUSSION

Colon cancer is a common malignancy[15,16]. With the

Table 1 Primers used for quantitative polymerase chain reaction

Primer	Sequence
HOTAIR	
Forward	5'-CAGTGGGGAACTCTGACTCG-3'
Reverse	5'-GTGCCTGGTGCTCTCTTACC-3'
GAPDH	
Forward	5'-GTCAACGGATTTGGTCTGTATT-3'
Reverse	5'-AGTCTTCTGGGTGGCAGTGAT-3'

Table 2 Relationship between HOTAIR expression and clinicopathological parameters in colon cancer

Clinicopathological parameter	No. of cases	HOTAIR expression	*P* value
Age (yr)			
< 6	49	3.69 ± 1.94	0.188
⩾ 60	31	3.45 ± 1.55	
Gender			
Male	43	3.91 ± 1.85	0.761
Female	37	3.23 ± 1.68	
Tumor size (cm)			
< 7	38	3.59 ± 1.59	0.599
⩾ 7	42	3.60 ± 1.81	
Lymph node metastasis			
Yes	48	4.27 ± 1.54	0.024
No	32	3.11 ± 1.92	
Tumor differentiation			
High and moderate	41	2.93 ± 1.62	0.019
Low and undifferentiated	39	4.35 ± 1.82	
TNM stage			
Ⅰ + Ⅱ	46	3.17 ± 1.77	0.034
Ⅲ + Ⅳ	34	3.87 ± 1.66	

Table 3 Cox multiple regression analysis of risk factors for prognosis of colon cancer patients

Variable	Regression coefficient	SE	χ^2	*P* value	OR	95%CI for OR	
						Lower	Upper
TNM stage	0.732	0.345	4.489	0.034	2.0790	1.056	4.090
Lymph node metastasis	-2.512	1.088	5.325	0.021	0.0081	0.010	0.685
HOTAIR expression	-2.048	0.785	6.806	0.090	0.1290	0.028	0.601

development of diagnostic technology, advances in endoscopy and imaging techniques, as well as the clinical application of the carcinoembryonic antigen assay, have greatly improved the early diagnosis and treatment effect of colon cancer[17,18]. Patients with early colon cancer have localized lesions, and surgery with adjuvant radiochemotherapy is the preferred treatment, which is often associated with a good prognosis. However, because of the unbalanced regional development in China, many patients with colon cancer, especially those in rural regions, are diagnosed at advanced stages, and some patients even present with metastases as the first manifestation. Although drugs targeting EGFR and

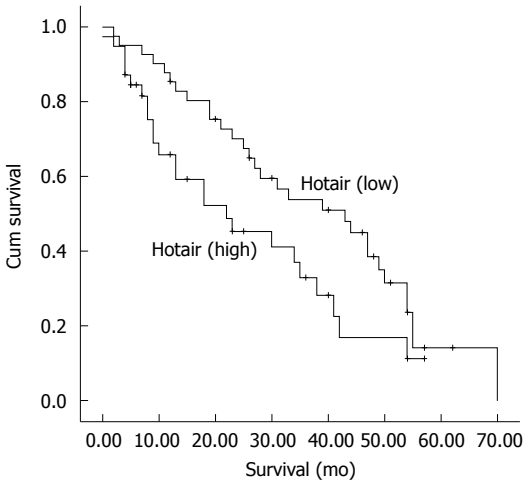

Figure 3 Relationship between HOTAIR expression and survival in colon cancer. The cumulative survival rate was significantly higher in patients with low HOTAIR expression than in those with high HOTAIR expression ($P < 0.05$).

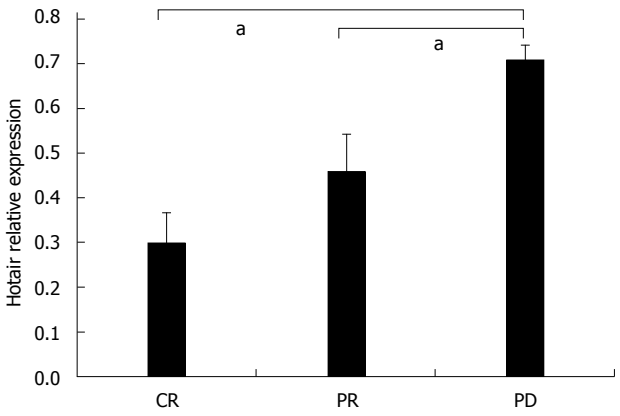

Figure 4 Relationship between HOTAIR expression and prognosis in colon cancer. [a]$P < 0.05$ *vs* PD group.

KRAS mutations have been effective in some patients with colon cancer[1-3], molecular targeted drugs, which often target only one or several molecules, are not suitable for all patients because of the complexity etiology of colon cancer. Therefore, there is an urgent need to find new therapeutic targets.

LncRNAs are non-protein coding transcripts of around 200 nucleotides that are widely distributed in the genome. Many lncRNAs can bind to DNA binding proteins and alter the chromosome state to participate in the regulation of many genes[6,19]. HOTAIR is an lncRNA located in the *HOXC* locus, and it can interact with polycomb repressive complex 2 and mediate the histone H3 lysine 27 methylation and lysine 4 demethylation in the *HOXD* locus, in which EZH2 also plays an important role[9,20,21]. HOTAIR can alter the state of chromosomes, thus affecting the expression of many genes. Researchers have found that HOTAIR expression is upregulated in cancer tissue samples from patients with breast cancer, pancreatic cancer, liver cancer, gastric cancer, or non-small cell lung cancer, and the expression is even higher in metastatic tissue.

Both *in vivo* and *in vitro* studies have confirmed that upregulated expression of HOTAIR enhances the ability of tumors to invade and metastasize[7-9]. The aim of this study was to detect the expression of HOTAIR in tissue samples from patients with colon cancer, analyze the relationship between HOTAIR expression and clinicopathological parameters and explore the role of HOTAIR in colon cancer development and metastasis.

The results showed that the expression of HOTAIR is upregulated in colon cancer, suggesting that HOTAIR may act as an oncogene in the development of colon cancer. We also discovered that HOTAIR expression was significantly higher in lowly differentiated and undifferentiated cases compared with highly and moderately differentiated cases; in stages Ⅲ + Ⅳ cases compared with stages Ⅰ + Ⅱ cases; and in cases with lymph node metastasis compared with those without. These results are similar to the findings of a previous study[22]; however, that study found that the expression of HOTAIR did not differ significantly between cases with and without lymph node metastasis, but was significantly higher in cases with liver metastasis compared with those without. The present study did not compare the HOTAIR expression between cases with and without liver metastasis. Low differentiation, late stage or lymph node metastasis in colon cancer are often associated with poor prognosis; therefore, our findings need to be validated by studies with a larger sample size.

Although HOTAIR might affect response to therapy in some tumors; for example, HOTAIR is associated with resistance to chemotherapy in ovarian cancer and sarcoma[23,24], there have been no reports in colon cancer. Our study, together with previous research, found that HOTAIR has an impact on the biological behavior of colon cancer[13], and detecting the level HOTAIR in blood could be used to predict prognosis of colon cancer. We speculated that this finding may be related to the role of HOTAIR in chemotherapy resistance, and this, therefore, was the focus of this study. We found that tumors with high expression of HOTAIR tended to develop resistance to chemotherapy, which may be the reason that high expression of HOTAIR is associated with a poor prognosis.

This finding also suggested that it is essential to explore the relationship between HOTAIR and resistance to chemotherapy *in vitro*, as well as the impact of HOTAIR on the biological behavior of tumor cells. Several studies have revealed that HOTAIR has an important role in tumor metastasis. On the basis of these findings, our subsequent follow-up study will expand the sample size to conduct prognostic and survival analyses to further define the relationship between HOTAIR and tumor metastasis in colon cancer, to explore the mechanism of pathogenesis of this malignancy and provide new targets for molecular therapy for colon cancer patients.

COMMENTS

Background

Long noncoding RNAs are non-protein coding transcripts of around 200 nucleotides that are widely distributed in the genome.

Research frontiers

The expression of HOTAIR, a long noncoding RNA, is upregulated in cancer tissue samples from patients with breast cancer, pancreatic cancer, liver cancer, gastric cancer, or non-small cell lung cancer, and the expression is even higher in metastatic tissue.

Innovations and breakthroughs

High expression of HOTAIR tended to develop resistance to chemotherapy, which may be the reason that high expression of HOTAIR is associated with a poor prognosis.

Applications

By exploration of the mechanism of HOTAIR expression in colon cancer, the authors might identify new targets for molecular therapy for colon cancer patients.

Terminology

HOTAIR might acts as an oncogene and represents a new molecular target for the treatment of colon cancer.

Peer-review

It is a very good and interesting study. The authors found that the expression of HOTAIR was significantly higher in colon cancer tissues than in matched tumor-adjacent normal colon tissues. HOTAIR expression was significantly higher in cases with lymph node metastasis than in those without metastasis, in lowly differentiated and undifferentiated cases than in highly and moderately differentiated cases. HOTAIR expression is upregulated in colon cancer, which may plays an important role in tumorigenesis, development and metastasis of colon cancer.

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ORIGINAL ARTICLE

Clinical Trials Study

Beneficial effects of antidepressant mirtazapine in functional dyspepsia patients with weight loss

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Abstract

AIM: To explore the effects and mechanism of action of antidepressant mirtazapine in functional dyspepsia (FD) patients with weight loss.

METHODS: Sixty depressive FD patients with weight loss were randomly divided into a mirtazapine group (MG), a paroxetine group (PG) or a conventional therapy group (CG) for an 8-wk clinical trial. Adverse effects and treatment response were recorded. The Nepean Dyspepsia Index-symptom (NDSI) checklist and the 17-item Hamilton Rating Scale of Depression (HAMD-17) were used to evaluate dyspepsia and depressive symptoms, respectively. The body composition analyzer was used to measure body weight and fat. Serum hormone levels were measured by ELISA.

RESULTS: (1) After 2 wk of treatment, NDSI scores were significantly lower for the MG than for the PG and CG; (2) After 4 or 8 wk of treatment, HAMD-17 scores were significantly lower for the MG and PG than for the CG; (3) After 8 wk of treatment, patients in the MG experienced a weight gain of 3.58 ± 1.57 kg, which was significantly higher than that observed for patients in the PG and CG. Body fat increased by 2.77 ± 0.14

kg, the body fat ratio rose by 4%, and the visceral fat area increased by 7.56 ± 2.25 cm^2; and (4) For the MG, serum hormone levels of ghrelin, neuropeptide Y (NPY), motilin (MTL) and gastrin (GAS) were significantly upregulated; in contrast, those of leptin, 5-hydroxytryptamine (5-HT) and cholecystokinin (CCK) were significantly downregulated.

CONCLUSION: Mirtazapine not only alleviates symptoms associated with dyspepsia and depression linked to FD in patients with weight loss but also significantly increases body weight (mainly the visceral fat in body fat). The likely mechanism of mirtazapine action is regulation of brain-gut or gastrointestinal hormone levels.

Key words: Mirtazapine; Functional dyspepsia; Weight loss; Depression

Core tip: A part of functional dyspepsia (FD) patients were found with weight loss in recent studies. As an antidepressant, mirtazapine was found not only to alleviate symptoms associated with dyspepsia and depression linked to FD with weight loss, but also to significantly increase body weight (mainly the visceral fat in body fat). Moreover, the likely mechanism of mirtazapine action is the regulation of brain-gut or gastrointestinal hormone levels.

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INTRODUCTION

Functional dyspepsia (FD) is a common clinical syndrome characterized by chronic and recurrent symptoms in the gastroduodenal region in the absence of any organic or metabolic disease that explains the symptoms[1]. It impairs the patient's quality of life and work efficiency, and increases the utilization of medical resources[2-4]. Psychosocial factors may play an important role in FD and lead to the use of antidepressant and anxiolytic agents in FD management[5].

Weight loss is a common symptom of digestive diseases, and may indicate an organic disease[6]. However, the indicators of weight loss for the diagnosis of an organic disease are limited[7-9]. Tack *et al*[10] found that of 40 FD patients, 55% had a weight loss that was > 5% of initial body weight. In a study investigating laboratory parameters and the nutritional status of 180 patients who were diagnosed with FD, 16.67% of patients had a weight loss from 5% to 10% of their initial body weight, and 4.44% had a weight loss of > 10% of their initial body weight[11]. Our previous multi-center research study of 1057 FD patients showed that with the onset of dyspepsia symptoms, 19.58% had lost ⩾ 5% of their initial body weight during the previous 12 mo or less. FD patients with weight loss had lower body mass index, more frequent physician visits, higher psychological disorders, poorer appetite and lower quality of life[12].

Antidepressant mirtazapine is clinically used in the treatment of depression or anxiety disorders. In recent years, many clinical trials associated with antidepressants for FD have indicated that antidepressants are effective in treating FD patients[13-15]. A case report about mirtazapine in the treatment of an FD patient with depression reported that the patient's indigestive symptoms, appetite, depression, and quality of life were improved after taking mirtazapine for 4 wk[16]. However, studies that have focused on the parts of the body that are involved in weight gain and the underlying mechanisms have been rare.

Clinical observations have shown that increases in appetite and food intake, and consequent weight gain occur in some patients undergoing mirtazapine treatment. Whereas such side effects may limit the general application of mirtazapine in antidepressant therapy, these very same effects proved to be beneficial in treating FD patients with weight loss.

Therefore, expanding upon previous work[12,17], in this study we comprehensively explored the effect of mirtazapine on depressive FD patients with weight loss by dynamic observation not only of the changes in dyspepsia and depressive symptoms but also the modifications of body weight and fat distribution and the levels of serum hormones.

MATERIALS AND METHODS

Ethics statement

This study was a prospective, randomized, controlled trial in depressive FD patients with weight loss and was approved by the hospital ethics committee (Clinical trial registration number: ChiCTR-TRC-13003161). Written informed consent was obtained from the patients according to the Declaration of Helsinki.

Patients

In this prospective study, 60 patients were recruited between September 2011 and June 2013 from the gastroenterology outpatient clinic of Guangzhou Nansha Central Hospital. All the patients fulfilled the following criteria[12]: (1) diagnosed with FD according to Rome Ⅲ criteria; (2) with a weight loss of ⩾ 5% of initial body weight since the onset of symptoms; (3) diagnosed with depression by psychiatrists according to the Chinese Classification of Mental Disorders (CCMD-3)

and scores of the Hamilton Rating Scale of Depression (HAMD) over 18; and (4) ranged in age from 18 to 65 years; and (5) signed informed consent statements.

The following exclusion criteria were adopted: (1) organic diseases such as peptic ulcers, atrophy or erosive gastroduodenal lesions, tumors, and esophagitis by gastroscopic examination; (2) liver, gallbladder, pancreas, spleen and bowel organic disease by laboratory, B ultrasonic or X-ray examination; (3) dyspepsia symptoms and weight loss that were explained by metabolic or infectious diseases such as diabetes, hyperthyroidism, or tuberculosis; (4) anorexia nervosa and patients with body weight management problems; (5) age < 18 or > 65 years; (6) pregnancy or breast feeding; (7) disabilities; (8) current use of other drugs in clinical research or use of similar drugs in the last half-month; (9) in a severe anxiety or depressive state, or with suicidal tendencies; (10) current use of non-steroidal anti-inflammatory drugs, steroids, or drugs affecting gastric acid secretion; and (11) contraindications for paroxetine or mirtazapine use including hypersensitivity, liver dysfunction or renal failure.

Grouping

Sixty depressive FD patients with weight loss were randomly divided into a mirtazapine group (MG), a paroxetine group (PG) or a conventional therapy group (CG) with 20 patients in each. The trial period spanned 8 wk. The CG was treated with histamine type 2 receptor antagonists or proton pump inhibitors or prokinetic agents; MG was treated with mirtazapine (Remeron®, N.V. Organon, Holland, 30 mg/d); and PG was treated with paroxetine (Seroxat®, SK&F, China, 20 mg/d). All protocols were based on conventional therapy.

Assessments

Adverse effects and treatment response were recorded and data collected at specific time points. These were before treatment (for baseline determination), 2 wk, 4 wk, 6 wk and 8 wk of treatment for the following assessments: dyspepsia symptoms were evaluated with NDSI; depressive symptoms, with HAMD-17; and the change in body weight and the distribution of body fat with the body composition analyzer (InBody720, Biospace, South Korea). Serum hormone levels were measured at baseline, 4 wk and 8 wk; expression levels of ghrelin, leptin, neuropeptide Y (NPY), 5-hydroxy tryptamine (5-HT), cholecystokinin (CCK), motilin (MTL) and gastrin (GAS) were assayed by ELISA.

NDSI: The NDSI evaluated the frequency, intensity, and practical impediments of 15 GI symptoms (including epigastric pain, epigastric burning, postprandial fullness, and early satiety) over a 2-wk period. We recorded each subscale score concerning daily activities/work (13 items), knowledge and control (7 items), eating/drinking (3 items), and sleep disturbance (2 items)[18]. We added each item score within each subscale to produce a subscale score. Low scores indicate mild symptoms.

HAMD-17: The rating standards of the HAMD[19] were as follows: no depression (0-6), mild depression (7-17), moderate depression (18-24), and severe depression (> 25). A higher score indicates worse depression.

Body composition analyzer (InBody720): Patient requirements included: empty stomach, empty bladder, light clothes and no shoes for measurement in the early morning on the body composition analyzer. Patients with a pacemaker or with metal in the body were excluded from measurement on this instrument.

Treatment response: Treatment response was defined as a > 50% reduction in the NDSI score. The response was calculated as: [(score at treatment - score at baseline)/score at baseline] × 100. The treatment responses of the three groups were calculated independently.

Statistical analysis

Data analyses were performed using SPSS 13.0 software (SPSS Inc., Chicago IL, United States), and measurement data are reported as the mean ± SD, and were compared across groups using one-way ANOVA and the Student-Newman-Keuls test for multiple comparisons. Count data were compared across groups using a χ^2 test. All tests were two-tailed and $P < 0.05$ was considered statistically significant.

RESULTS

Study participants

A total of 60 depressive FD patients with weight loss were enrolled in the study. All patients were randomized to receive mirtazapine, paroxetine or conventional treatment. No patient was lost to follow-up. The baseline characteristics of the patients are shown in Table 1. No differences were observed among the three groups in gender, age, height, weight, body mass index (BMI) or body loss when diagnosed.

Improvement of dyspepsia symptoms

As shown in Figure 1A, the patients' dyspepsia symptoms gradually improved over the course of treatment in the three groups. After 2 wk, the NDSI score was significantly lower in the MG than in the PG and CG ($P < 0.05$ for all), and this trend continued until the end of the study. Since 6 wk, NDSI score of the PG was significantly lower than that of the CG ($P < 0.05$).

Improvement of depressive symptoms

Patients' depressive symptoms were improved in the

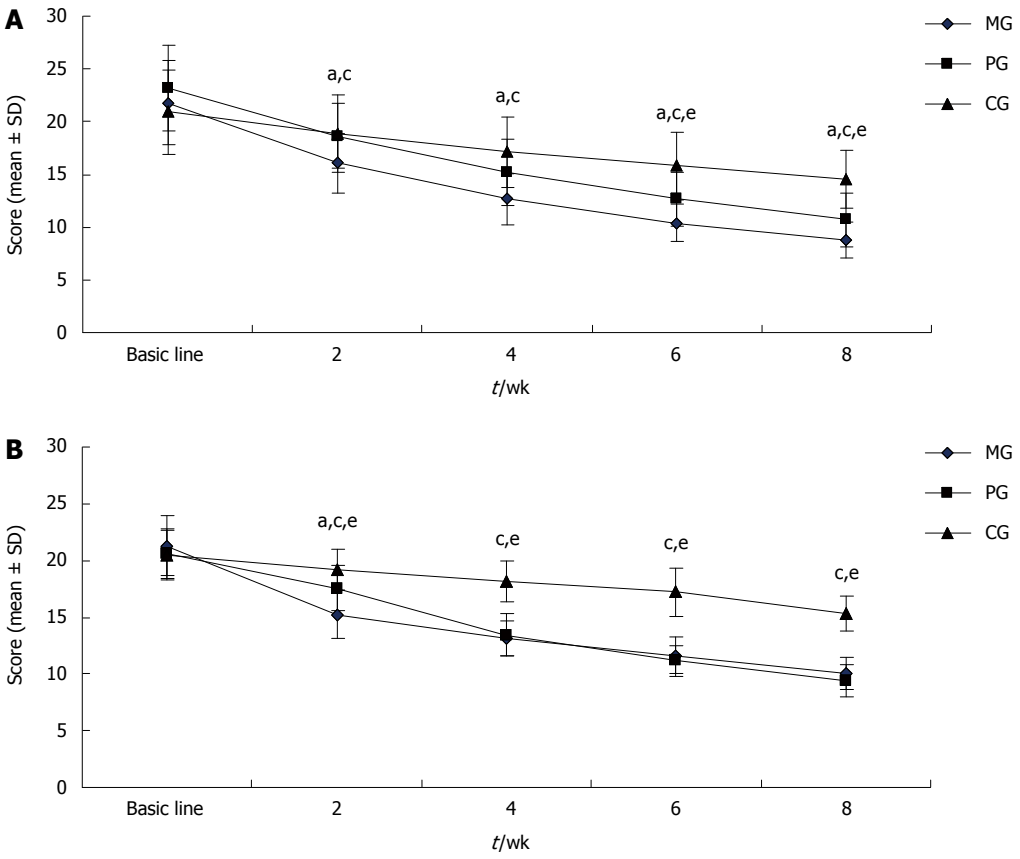

Figure 1 Comparison of Nepean Dyspepsia Index-symptom scores (A) or Hamilton Rating Scale of Depression-17 scores (B). [a]*P* < 0.05, MG *vs* PG; [c]*P* < 0.05, MG *vs* CG; [e]*P* < 0.05, PG *vs* CG. MG: Mirtazapine group; PG: Paroxetine group; CG: Conventional group.

Table 1 General characteristics of study patients

Variable	MG (*n* = 20)	PG (*n* = 20)	CG (*n* = 20)	*P* value
Gender (M/F)	8/12	11/9	7/13	0.725
Age (yr)	43.45 ± 11.50	37.75 ± 10.78	39.95 ± 6.84	0.914
Height (cm)	163.81 ± 12.36	162.36 ± 10.06	160.72 ± 10.63	0.834
Weight (kg)	49.77 ± 6.79	48.93 ± 5.89	48.22 ± 5.57	0.973
BMI (kg/m^2)	18.73 ± 5.62	18.65 ± 4.73	18.84 ± 6.38	1.005
Body loss when diagnosed	3.42 ± 0.54	3.72 ± 0.64	3.69 ± 0.71	0.872

MG: Mirtazapine group; PG: Paroxetine group; CG: Conventional group.

three treatment groups (Figure 1B). At all time points, the HAMD-17 score was significantly lower in the MG and PG than in the CG ($P < 0.05$). After 2 wk of treatment, the HAMD-17 score was sharply lower in the MG than in the PG ($P < 0.05$); however, at 4 wk, the score of PG became very close to that of the MG and remained so until the end of the study.

Change of body weight and its composition

As shown in Figure 2, the patients' body weights were not significantly different before treatment among the three groups. As treatment progressed, body weight of patients in the MG gradually increased. At 6 wk and 8 wk, patients' body weights were significantly heavier in the MG than those in the PG and CG ($P < 0.05$ for all); thus, there was no significant body weight change over the test period in either the PG or CG.

Further analysis of body weight and its composition is shown in Table 2. After 8 wk of treatment, 19 patients in the MG presented an increase in body weight and BMI. The patients in the MG gained 3.58 ± 1.57 kg, which was significantly higher than that gained in either the PG, at 0.53 ± 0.44 kg or the CG, at 0.56 ± 0.45 kg ($P < 0.05$). However, no obvious change of body weight was observed in the PG or CG throughout treatment. Body fat is one of the main components contributing to body weight. Over the course of treatment, body fat increased by 2.77 ± 0.14 kg; the body fat ratio rose by 4%; and visceral fat area was increased by 7.56 ± 2.25 cm^2 ($P < 0.05$). No significant change in muscle volume was detected over the treatment period.

Changes in expression levels of serum hormones

After 4 wk and 8 wk of treatment in MG, the levels of ghrelin, NPY, MTL, and GAS were significantly upregulated, while the levels of leptin, 5-HT and CCK were significantly downregulated ($P < 0.05$). After 8 wk of treatment, significant differences appeared between the levels in the MG and those in the PG and CG ($P < 0.05$). Moreover, at 4 wk in the PG, the levels of NPY, MTL and GAS sharply increased, whereas

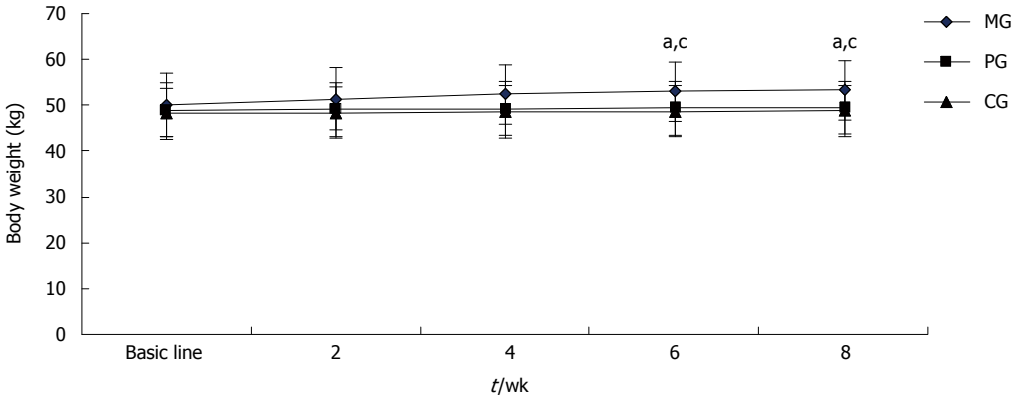

Figure 2 Change of body weight. [a]*P* < 0.05, MG *vs* PG; [c]*P* < 0.05, MG *vs* CG. MG: Mirtazapine group; PG: Paroxetine group; CG: Conventional group.

Table 2 Change of body weight and its composition in mirtazapine group

Variable	Baseline	2 wk	4 wk	6 wk	8 wk
Body weight (kg)	49.77 ± 6.79	51.35 ± 6.80	52.36 ± 6.60[a]	53.07 ± 6.46[a]	53.35 ± 6.52[a]
BMI (kg/m^2)	18.73 ± 5.62	18.97 ± 5.43	19.70 ± 4.52[a]	19.87 ± 4.62[a]	20.07 ± 5.23[a]
Body fat (kg)	8.36 ± 2.53	9.28 ± 2.33	11.05 ± 1.92[a]	11.09 ± 1.87[a]	11.13 ± 2.86[a]
Body fat ratio (%)	0.17 ± 0.02	0.18 ± 0.04	0.20 ± 0.07[a]	0.20 ± 0.09[a]	0.21 ± 0.08[a]
Visceral fat area (cm^2)	35.27 ± 8.12	36.12 ± 8.05	41.68 ± 9.23[a]	42.02 ± 9.14[a]	42.83 ± 10.64[a]
Muscle volume (kg)	38.25 ± 7.53	38.26 ± 7.49	38.46 ± 6.84	38.52 ± 6.64	38.62 ± 6.77

[a]*P* < 0.05 *vs* baseline. BMI: Body mass index.

Table 3 Changes of expression level of serum hormones

Group		Ghrelin (ng/mL)	Leptin (ng/mL)	NPY (pg/mL)	5-HT (ng/mL)	CCK (pg/mL)	MTL (pg/mL)	GAS (pg/mL)
MG	Baseline	4.62 ± 1.53	9.87 ± 4.65	107.52 ± 26.21	237.83 ± 56.94	392.36 ± 27.21	32.53 ± 6.28	56.37 ± 21.15
	4 wk	6.02 ± 3.43[a,c,e]	7.25 ± 3.47[a,c,e]	114.56 ± 25.10[a]	208.47 ± 52.48[a]	221.69 ± 23.77[a]	49.46 ± 6.10[a,c,e]	48.37 ± 11.93[a,c,e]
	8 wk	8.97 ± 3.64[a,c,e]	4.03 ± 2.77[a,c,e]	149.27 ± 39.53[a,c,e]	176.92 ± 53.38[a,c,e]	183.85 ± 27.65[a,c,e]	66.28 ± 3.97[a,c,e]	41.61 ± 10.52[a,c,e]
PG	Baseline	4.68 ± 2.12	9.07 ± 4.65	105.12 ± 29.52	232.83 ± 50.94	390.82 ± 27.54	31.98 ± 9.34	54.98 ± 15.24
	4 wk	5.12 ± 2.23	8.75 ± 3.05	112.31 ± 15.10[a]	215.32 ± 24.91[a]	291.57 ± 31.76[a]	40.37 ± 8.23[a]	52.37 ± 12.97
	8 wk	6.01 ± 3.27[a]	8.25 ± 2.13	114.27 ± 28.53[a]	210.17 ± 49.17[a]	283.85 ± 47.15[a]	53.28 ± 6.84[a]	48.61 ± 11.17[a]
CG	Baseline	4.89 ± 2.47	8.87 ± 2.65	104.93 ± 17.95	235.81 ± 61.82	391.75 ± 24.96	36.26 ± 6.22	54.23 ± 26.83
	4 wk	5.48 ± 2.15	8.32 ± 3.57	108.92 ± 29.64	211.26 ± 46.28	224.67 ± 23.45	43.92 ± 7.24	49.21 ± 12.15
	8 wk	6.93 ± 2.35	8.02 ± 1.45	121.43 ± 13.92	208.95 ± 38.29	303.12 ± 26.76	55.53 ± 5.98	43.34 ± 13.72

[a]*P* < 0.05 *vs* baseline in the same group; [c]*P* < 0.05, MG *vs* PG; [e]*P* < 0.05, MG *vs* CG. MG: Mirtazapine group; PG: Paroxetine group; CG: Conventional group; NPY: Neuropeptide Y; 5-HT: 5-hydroxytryptamine; CCK: Cholecystokinin; MTL: Motilin; GAS: Gastrin.

the levels of 5-HT and CCK decreased. There was no obvious difference in CG hormone expression levels over the treatment period (Table 3).

Adverse effects and treatment response

The adverse effects associated with the different protocols were recorded for the 20 patients assigned to each treatment group**:** in the MG, these were dizziness (10%), lethargy (15%), and fatigue (15%); in the PG, they were dizziness (15%), lethargy (20%), nausea (5%) and fatigue (20%). As these adverse effects were mild, they dissipated without treatment within 1 wk. No obvious adverse effects were reported in the CG.

After 8 wk, 85% of patients in the MG, and 80% in the PG responded positively to treatment, which were significantly higher than that (55%) found in the CG; however, there was no significant difference in the results between the MG and PG.

DISCUSSION

FD is a common psychosomatic disease associated with a variety of mental disorders including anxiety, depression, panic attacks, and post-traumatic stress disorder, of which anxiety and depression are the most common. Negative spiritual, psychological and social factors can accelerate the onset of FD symptoms and exacerbate them and thereby ultimately affect treatment efficacy. However, at present, the impact of such factors on the incidence and progression of FD is not very clear; one intriguing possibility is that

they may work to change gastrointestinal motor or sensory function through the brain-gut axis. Weight loss is a common symptom of digestive diseases, and may indicate an organic disease[6], but recently, certain studies have found that patients with functional gastrointestinal diseases often showed weight loss[11,20].

Currently, there is no very effective treatment for depressive FD patients with weight loss, because of the chronic and recurrent characteristics of the disease. Antidepressants are often used to treat patients with depression. One of these, mirtazapine, a serotonin-norepinephrine reuptake inhibitor that is clinically used for the treatment of depression, acts rapidly with positive effects on sleep disorders, appetite loss, depressive symptoms, *etc*.

Herein, we analyzed the effects of mirtazapine on depressive FD patients with weight loss**.** Mirtazapine showed higher efficacy in relieving dyspeptic symptoms and lowering NDSI scores when compared to paroxetine and conventional treatment, and was equal to paroxetine in mitigating depressive symptoms. After 8 wk of treatment, 85% of MG patients were classified as treatment responsive, a proportion higher than 80% as observed in the PG, and significantly higher than 55% as seen in the CG. This may be related to specific aspects of mirtazapine action that not only may alleviate depression and improve the function of the nervous system, but also regulate gastrointestinal motor or sensory function.

In this study, we show that mirtazapine treatment of depressive FD patients with weight loss not only effectively treated symptoms of dyspepsia and depression, but also induced significant weight gain, an effect not observed with either paroxetine or conventional treatment. Specifically, 80% of the patients experienced weight gain after 4 wk of treatment with mirtazapine; furthermore, 95% of these patients continued to gain weight until the end of the treatment. The average weight gain was 3.58 ± 1.57 kg, resulting in significantly higher weight than the baseline weight recorded before treatment. In humans, weight is mainly composed of muscle volume, body fat, and inorganic salts, and muscle volume and body fat are the most affected. Through dynamic observation of the weight distribution of the various body components, we found that muscle volume stayed relatively constant throughout treatment, whereas body fat significantly changed. Body fat, which includes subcutaneous fat, visceral fat, muscle clearance fat, proved to be the main contributor to body weight gain. Further analysis of body fat distribution revealed that visceral fat showed a marked increase with mirtazapine treatment at 4 wk and 8 wk, which indicated that visceral fat was the key element responsible for the observed body weight gain.

Generally, significant imbalances of visceral fat are known to increase the incidence of cardiovascular disease, digestive disease, urinary disease, *etc*. In our study, although visceral fat did indeed increase after 8 wk of mirtazapine treatment, body fat ratios remained at normal levels. We speculate that most of the patients were at a low level of body weight and BMI before treatment, and even underweight according to BMI, whereas muscle volume remained at normal levels throughout; thus, the amount of body fat must have been seriously deficient before treatment. Moreover, through appetite growth and symptom relief, muscle volume may have gradually increased with treatment, whereas overall body fat may have grown at a slower rate.

In recent years, functional gastrointestinal disease has been closely associated with dysregulation of the brain-gut axis. The brain-gut axis, which is regulated by neuroendocrine and immune factors, is a bipolar system between the gastrointestinal tract and brain that is affected by psychosocial factors. The coordination between the central nervous system and gastrointestinal contractility is regulated through a variety of brain-gut peptides and gastrointestinal hormones. In this study, the levels of ghrelin, NPY, MTL, and GAS which may increase appetite, food intake or gastrointestinal dynamic promotion were significantly upregulated, whereas the levels of leptin, 5-HT and CCK which may decrease food intake, block gastrointestinal motility or increase gastrointestinal sensitivity were significantly downregulated.

In conclusion, antidepressant mirtazapine not only improved patients' conditions concerning indigestive and depressive symptoms, but also increased appetite and body weight (mainly the visceral fat in body fat), much more effectively than either paroxetine or conventional therapy. The clinical efficacy of mirtazapine may be mediated in part through the regulation of brain-gut or gastrointestinal hormones. To clarify these effects and the underlying mechanisms of mirtazapine action in FD patients with weight loss will require bigger sample sizes, and multi-center, randomized controlled trials in future studies.

COMMENTS

Background

Functional dyspepsia (FD) is a common psychosomatic disease associated with a variety of mental disorders, and weight loss was often found in FD patients. Such patients had lower body mass index, more frequent physician visits, higher psychological disorders, poorer appetite and lower quality of life. In recent years, many clinical trials indicated that antidepressant mirtazapine are effective in treating FD patients. Whereas some side effects may limit the general application of mirtazapine in antidepressant therapy, these may prove to be beneficial in treating FD patients with weight loss.

Research frontiers

This study comprehensively explored the effect of mirtazapine on depressive FD patients with weight loss by dynamic observation not only of the changes in dyspepsia and depressive symptoms but also the modifications of body weight and fat distribution and the level of serum hormones.

Innovations and breakthroughs

This study showed that antidepressant mirtazapine not only improved patients' conditions concerning indigestive and depressive symptoms, but also increased appetite and body weight, mainly the visceral fat in body fat, much more effectively than either paroxetine or conventional therapy. The clinical efficacy of mirtazapine may be mediated in part through the regulation of brain-gut or gastrointestinal hormones.

Applications

The findings can supply the evidence for the clinical application of mirtazapine in FD patients with weight loss.

Terminology

The Nepean Dyspepsia Index-symptom is a scale that evaluates the frequency, intensity, and practical impediments of 15 gastrointestinal symptoms.

Peer-review

This is a good and practical study in which the authors found that the beneficial effects and mechanism of action of antidepressant mirtazapine in FD patients with weight loss. It is believed that the findings can provide a new angle and evidence for the clinical application of mirtazapine in FD patients with weight loss.

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ORIGINAL ARTICLE

Observational Study

Inflammatory bowel disease: A descriptive study of 716 local Chilean patients

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Abstract

AIM: To demographically and clinically characterize inflammatory bowel disease (IBD) from the local registry and update data previously published by our group.

METHODS: A descriptive study of a cohort based on a registry of patients aged 15 years or older who were diagnosed with IBD and attended the IBD program at Clínica Las Condes in Santiago, Chile. The registry was created in April 2012 and includes patients registered up to October 2015. The information was anonymously downloaded in a monthly report, and the information on patients with more than one visit was updated. The registry includes demographic, clinical and disease characteristics, including the Montreal Classification, medical treatment, surgeries and hospitalizations for crisis. Data regarding infection with *Clostridium difficile*

(*C. difficile*) were incorporated in the registry in 2014. Data for patients who received consultations as second opinions and continued treatment at this institution were also analyzed.

RESULTS: The study included 716 patients with IBD: 508 patients (71%) were diagnosed with ulcerative colitis (UC), 196 patients (27%) were diagnosed with Crohn's disease (CD) and 12 patients (2%) were diagnosed with unclassifiable IBD. The UC/CD ratio was 2.6/1. The median age was 36 years (range 16-88), and 58% of the patients were female, with a median age at diagnosis of 29 years (range 5-76). In the past 15 years, a sustained increase in the number of patients diagnosed with IBD was observed, where 87% of the patients were diagnosed between the years 2001 and 2015. In the cohort examined in the present study, extensive colitis (50%) and colonic involvement (44%) predominated in the patients with UC and CD, respectively. In CD patients, non-stricturing/non-penetrating behavior was more frequent (80%), and perianal disease was observed in 28% of the patients. There were significant differences in treatment between UC and CD, with a higher use of corticosteroids, and immunosuppressive and biological therapies was observed in the patients with CD ($P < 0.05$ and $P < 0.01$). Significant surgical differences were also observed: 5% of the UC patients underwent surgery, whereas 38% of the CD patients required at least one surgery ($P < 0.01$). The patients with CD were hospitalized more often during their disease course than the patients with UC (55% and 35% of the patients, respectively; $P < 0.01$). *C. difficile* infection was acquired by 5% of the patients in each group at some point during the disease course. Nearly half of the patients consulted at the institution for a second opinion, and 32% of these individuals continued treatment at the institution.

CONCLUSION: IBD has continued to increase in the study cohort, slowly approaching the level reported in developed countries.

Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; South America; Latin America; Chile; Epidemiology

Core tip: Several studies have found that the incidence of inflammatory bowel disease (IBD) has increased over the past several decades, even in countries where the frequency was extremely low. Industrialization, increased physician awareness, advancements in diagnostic methods and better access to medical services are factors that might explain this increase. Although few epidemiological studies have been conducted in Latin America, these analyses have described an increased incidence of IBD. In the present study, we analyzed single-center data of 716 patients with IBD. We collected data from a considerable number of patients diagnosed with IBD, enabling the demographic and clinical characterization of these individuals.

Simian D, Fluxá D, Flores L, Lubascher J, Ibáñez P, Figueroa C, Kronberg U, Acuña R, Moreno M, Quera R. Inflammatory bowel disease: A descriptive study of 716 local Chilean patients. *World J Gastroenterol* 2016; 22(22): 5267-5275 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i22/5267.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i22.5267

INTRODUCTION

Inflammatory bowel disease (IBD) includes a spectrum of typically progressive chronic diseases, including Crohn's disease (CD), ulcerative colitis (UC) and unclassified colitis. Unclassified colitis occurs in patients who have clinical and endoscopic evidence of chronic IBD affecting the colon without small bowel involvement and no definitive histological or other evidence suggesting either CD or UC[1]. Although IBD mortality is low, the onset of this disease during early adulthood and its chronicity as a lifelong disease result in a significant decline in the quality of life of the patients and a heavy burden on the healthcare system due to high treatment costs[2]. Natural history studies have helped identify subsets of patients whose disease prognosis can be stratified according to clinical features. These data might improve the management of patients with IBD by defining changes in disease phenotype and risks of relapse, hospitalization and surgery[3].

Several studies have reported that the incidence of IBD has markedly increased over the latter part of the 20th century, whereas other studies have suggested a plateau or even a decline in IBD incidence in certain geographical areas[4,5]. However, an increase in these diseases has been described in countries where their frequency was very low[6-9]. IBD has been associated with the industrialization of nations[10,11], and thus, the increasing incidence of these diseases in developing countries might reflect this phenomenon. However, other factors, such as increased physician awareness, advancements in diagnostic methods and better access to medical services, such as colonoscopies, should be considered[12]. Although few epidemiological studies have been conducted in developing Latin American countries, these analyses have also described an increased incidence of IBD[13-19]. As we previously published, the incidence and prevalence of IBD in Chile are unknown; however, consistently with two other studies, our data suggest increases in the numbers of local cases of CD and UC[16,18,20]. The objective of this study was to demographically and clinically characterize IBD from a local registry and thereby update previously published data.

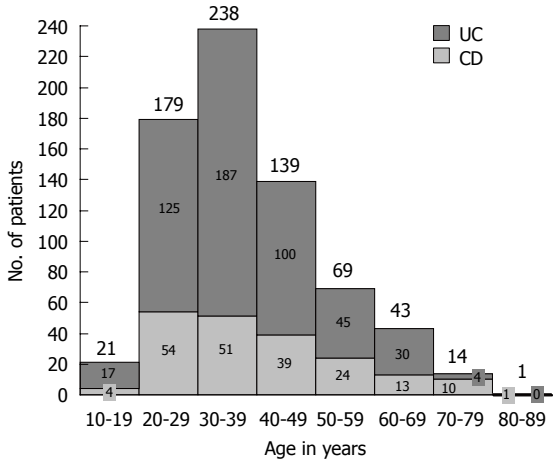

Figure 1 Frequency of patients with ulcerative colitis and Crohn´s disease at age of diagnosis. UC: Ulcerative colitis; CD: Crohn´s disease.

MATERIALS AND METHODS

This was a descriptive study of a cohort based on a registry of patients aged 15 years and older, diagnosed with IBD according to clinical, endoscopic, histological and radiologic findings and attending the IBD program at Clínica Las Condes in Santiago, Chile. The registry was created in April 2012 and includes patients who attended the program until October 2015. Retrospective data were obtained from those patients diagnosed prior to the indicated date. The registry is composed of online forms available in the electronic medical record of each patient that were completed by gastroenterologists and colorectal surgeons during clinic visits. On each subsequent visit, the information was prospectively updated as deemed necessary. The information was anonymously downloaded in a monthly report, and the information for patients with more than one total visit was updated. The registry includes demographic, clinical and disease characteristics, such as extension, location and behavior, changes in diagnosis from UC to CD, phenotype changes in CD, medical treatment, surgeries and hospitalizations for crisis. Data concerning *Clostridium difficile* (*C. difficile*) infection were incorporated into the registry in 2014. A polymerase-chain reaction assay for *C. difficile* detection was requested for patients presenting with moderate-to-severe activity. In CD, the Montreal Classification was used to define the phenotype as follows: B1, non-stricturing/non-penetrating; B2, stricturing; and B3, penetrating. A "p" was added to any of these classifications in case of perianal disease. The same classification was used to define the location of the disease: L1, ileum; L2, colon; L3, ileocolonic; and L4, concomitant upper gastrointestinal involvement. The extension of UC was defined according to the Montreal Classification: E1, ulcerative proctitis; E2, left-sided UC (distal UC); and E3, extensive UC (pancolitis)[1]. However, because Clínica Las Condes is a tertiary center that receives patients from locations throughout the country, the data for patients who received consultations as a second opinion and continued treatment at this institution were also analyzed. Patients with two or more visits over the next year were considered patients who were continuing treatment with the IBD program at this institution. This study was approved through the Institutional Ethics Committee.

Statistical analysis

The data were analyzed using the R Commander program. Continuous variables did not have a normal distribution and were described based on medians and ranges and compared using the Mann Whitney rank test for independent groups. Qualitative categorical variables were described with absolute frequency and percentage, and we used the χ^2 test for comparative statistical analysis. When the sample was less than 20, Fisher's exact test was used. Differences with a *P* value less than 0.05 were considered to be statistically significant. A biomedical statistician conducted a statistical review of the present study.

RESULTS

The study included 716 patients with IBD: 508 patients (71%) were diagnosed with UC, 196 patients (27%) were diagnosed with CD, and 12 patients (2%) were diagnosed with unclassifiable IBD. The UC/CD ratio was 2.6/1. The median age was 36 years (range 16-88), and 58% of the patients were female, with a median age at diagnosis of 29 years (range 5-76). Most patients with UC and CD were diagnosed between the ages of 20 and 29 years (Figure 1), without differences in gender. However, 22 patients (3%) were diagnosed when over 60 years of age. In the past 15 years, a sustained increase in the number of patients diagnosed with IBD has been observed, and significant increases were obtained from the comparison of the periods 1971-1985, 1986-2000 and 2001-2015, with 87% of patients diagnosed in the last period. The frequency of patients with IBD distributed according to the year of diagnosis is shown in Figure 2, illustrating an increase in the diagnosis of new cases of UC and CD over time. The demographic and disease characteristics of patients are shown in Table 1. In both UC and CD patients, articular symptoms were the most frequent extraintestinal manifestations. Primary sclerosing cholangitis (PSC) was diagnosed in eight patients (2%) with UC and two patients (1%) with CD.

Extent, location and behavior of IBD

Regarding the extent of UC, 50% of patients had extensive colitis. In CD, 44% of patients had colonic involvement, and only 3% of patients presented with concomitant upper disease. One patient in the registry presented with isolated perianal disease, and CD was confirmed through biopsies of the fistula,

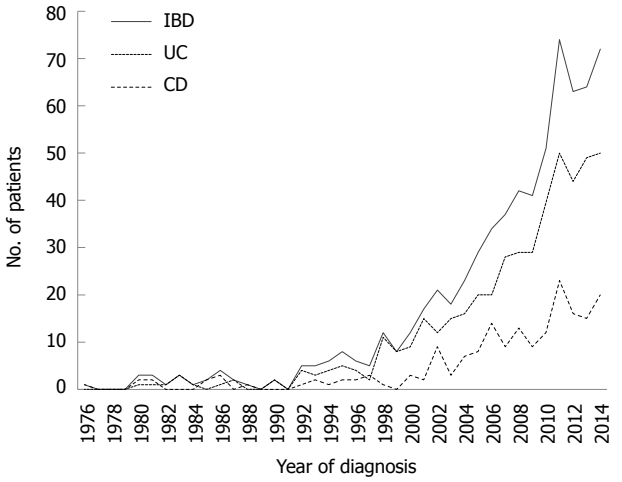

Figure 2 Frequency of patients with inflammatory bowel disease distributed by year of diagnosis. IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn´s disease.

Table 1 Demographic and disease characteristics *n* (%)

	UC (*n* = 508)	CD (*n* = 196)
Smoking habit		
Active	47 (9)	31 (16)
Discontinued	75 (15)	34 (17)
Family history of IBD	59 (12)	19 (10)
Extraintestinal manifestations		
Articular	156 (31)	87 (44)
Dermatological	11 (2)	10 (5)
Ocular	8 (2)	3 (2)
Other	31 (6)	15 (8)

UC: Ulcerative colitis; CD: Crohn´s disease.

which demonstrated granulomas. Non-stricturing/non-penetrating behavior was predominant in CD (80%). Stricturing and penetrating behavior was observed in 10% and 9% of patients, respectively. Perianal disease was observed in 28% of the patients with CD (Table 2). During the course of IBD, the diagnosis of 19 patients changed: one patient with unclassifiable IBD was newly diagnosed with CD, and 18 UC patients were newly diagnosed with CD. In six patients, CD was posteriorly diagnosed because these individuals developed perianal fistulas. In addition, 16 patients with CD showed modified behavior, nine of these patients showed changes from non-stricturing/non-penetrating to stricturing disease and seven of them showed changes from non-stricturing/non-penetrating to penetrating disease. In addition, two patients who are included in the 16 patients mentioned above developed perianal disease. Changes in disease extension were observed in 36 UC patients. Specifically, 12 of these 36 patients presented disease extension from proctitis to left colitis, and the remaining 24 patients exhibited disease extension from proctitis or left colitis to extensive colitis.

Medical treatment for IBD

Significant differences in treatment were found between

Table 2 Extent, location and behavior of inflammatory bowel disease according to the montreal classification *n* (%)

	UC (*n* = 508)	CD (*n* = 196)
UC Extent		
E1: Ulcerative proctitis	142 (28)	
E2: Left sided UC	112 (22)	
E3: Extensive UC (pancolitis)	254 (50)	
CD Location		
L1: Ileal		53 (27)
L2: Colonic		87 (44)
L3: Ileocolonic		55 (28)
L4: Upper gastrointestinal		5 (3)
CD Behavior		
B1: Non-stricturing/non-penetrating		157 (80)
B2: Stricturing		20 (10)
B3: Penetrating		18 (9)
p: Perianal disease		55 (28)

UC: Ulcerative colitis; CD: Crohn´s disease.

Table 3 Medical treatment, hospitalizations and surgery in patients with inflammatory bowel disease *n* (%)

	UC (*n* = 508)	CD (*n* = 196)	*P* value
Corticosteroids	297 (58)	133 (68)	< 0.05
Mesalazine (oral, local or both)	497 (98)	133 (68)	< 0.01
Immunosupressive agents	166 (33)	132 (67)	< 0.01
Ciclosporine	7 (1)	2 (1)	
Biologic therapy	34 (7)	67 (34)	< 0.01
Surgery	27 (5)	75 (38)	< 0.01
Intestinal resection	27 (5)	50 (25)	< 0.01
Hospitalizations	176 (35)	108 (55)	< 0.01
1	113 (64)	50 (46.3)	< 0.01
2-3	45 (26)	35 (32.3)	
⩾ 4	18 (10)	23 (21.3)	< 0.01[1]

[1]Fisher´s Test. UC: Ulcerative colitis; CD: Crohn´s disease.

UC patients and CD patients (Table 3). Mesalamine was used to treat 98% of UC patients and 68% of CD patients. Patients with CD received corticosteroids, mesalamine and immunosuppressive agents at equal frequency. A comparison of both groups revealed that the use of corticosteroids and immunosuppressive and biological therapies was significantly higher in patients with CD. A total of 102 patients (14%) were treated with biological therapy; specifically, 83 patients received infliximab, 13 patients received adalimumab, one patient received certolizumab pegol, one patient received golimumab and four patients received natalizumab. Biological therapy was initiated one year (median) after diagnosis for patients diagnosed since 2010 (39 patients) because biological therapy has become more accessible since then.

Surgery in IBD

A comparison revealed that more CD than UC patients required surgery. Specifically, only 27 UC patients (5%) underwent surgery, whereas 75 CD patients (38%) underwent surgery ($P < 0.01$) (Table 3). Fifty of the patients diagnosed with CD (25%) underwent intestinal resection, and six of these patients (3%)

required surgery for posteriorly based perianal disease. In addition, 24 CD patients (12%) underwent surgery due only to perianal disease, and one patient underwent a loop ileostomy.

Hospitalization and C. difficile infection

CD patients were hospitalized more often during the disease course than UC patients (55% and 35% of patients, respectively; $P < 0.01$). In addition, 21.3% of the CD patients had more than four hospitalizations compared with 10% of the UC patients ($P < 0.01$). The median number of hospitalizations for both was 2, with ranges from 1 to 24 for UC and 1 to 40 for CD (Table 3).

As previously described, data concerning *C. difficile* infection were incorporated into the registry in 2014, and a total of 490 patients (344 UC patients and 141 CD patients) were analyzed. No differences in the prevalence of infection was found between the groups, and 5% of the patients in each group acquired *C. difficile* infection at some point during the disease course.

Second opinion

The data obtained from the registry showed that 328 patients (46%) received consultations at the institution for a second opinion. Most of these patients (72%) were diagnosed with UC, and 107 of the 328 patients (32%) continued treatment at this center. However, 130 patients were not analyzed because these individuals received consultations late in 2015 and had therefore not completed more than one year of treatment since the first consultation at this institution. In addition, many patients live in other cities and receive consultations only for complex situations.

DISCUSSION

The number of patients in the registry more than doubled compared with the number detailed in our previous publication[20]. This increase not only allowed us to better characterize these patients but also facilitated a comparison with studies conducted worldwide. This population increase reflects not only the recognition of this institute as a referral center but also the increased rate of IBD diagnoses in recent years.

The cohort UC/CD ratio in 2014 was 2.9/1, and the current ratio has increased 2.6/1, showing a slow approaching to the ratio reported in developed countries (1/1)[6,13,21,22]. However, genetic, environmental and geographic factors may influence this difference. A gender-based analysis showed that the percentages of UC and CD were slightly higher in women, which is consistent with the results obtained by other series[13,14,17]. The median age at diagnosis was 29 years, and 64% of patients were diagnosed between 20 and 39 years of age. No second peak was observed at an older age, which is consistent with the results from recent studies[6,7,23-25].

An active smoking habit was almost twice as frequent in patients with CD (16%) compared with patients with UC (9%), regardless of whether the patients with CD received counseling and were told of the particular deleterious effect of smoking. This finding demonstrates that smoking education is important and that smoking cessation should be emphasized on every visit. Nonetheless, the frequencies of patients with active smoking habits observed in the present study were low compared with the frequency of active smokers in the general population. The last National Health Survey has demonstrated that 40.6% of the adult population smokes regularly, indicating that cigarette smoking is an important health problem in Chile and that other factors, such as environmental factors, may be influencing the increase in the diagnosis of CD[26].

A family history was obtained for 12% and 10% of the patients diagnosed with UC and CD, respectively, similarly to the results reported by Moller *et al*[27], who published a much larger study. The analysis of the clinical characteristics revealed that the most frequent extra-intestinal manifestation was articular symptoms, with frequencies of 31% and 44% in UC and CD patients, respectively. In previous studies, musculoskeletal manifestations were described as the most common extra-intestinal manifestation, and UC patients are more affected than CD patients. However, the percentage of affected patients was lower than that observed in the present study (20%-30%)[28]. It has been suggested that the risk of developing peripheral arthritis increases with an increase in the extent of IBD activity[29,30], and 70% of patients diagnosed with CD who showed musculoskeletal manifestations had either colonic or ileocolonic involvement, whereas 55% of the patients diagnosed with UC who showed musculoskeletal manifestations had pancolitis. This observation reflects the recognition of this institution as a tertiary referral center that receives complex patients. In addition, the etiology of peripheral arthritis in IBD might reflect a combination of genetic predisposition and exposition to the luminal bacterial bowel contents[30].

The analysis of the extent of UC showed that half of the patients had extensive colitis, different from the frequency described in previous studies, which reported that distal location predominates[9] and that extensive colitis varies between 20 and 40%[7,9,19,22,24,31]. Even a previous study published in Chile, which involved two different institutions, reported 38% and 15% extensive colitis; notably, the frequency of 15% was previously reported at our institution 10 years ago[18], before this institution was recognized as a referral center and before its association with an IBD program. The higher percentage of extensive colitis observed in the population examined in the present study might reflect the fact that it was conducted at a tertiary referral

center and included refractory cases that were difficult to treat.

Colonic involvement was more frequent in the CD patients examined in the present cohort, with a frequency of 44%. This finding is consistent with the results of a study conducted in Brazil, which found that colonic involvement predominates, although at a lower frequency (36%)[19]. Non-structuring/non-penetrating behavior was more frequently observed in the cohort examined in the present study, with a frequency of 80%. This value is consistent with the frequency described in previous studies, which showed that inflammatory phenotypes predominate during the first years of the disease[24]. During the disease course, approximately 10% of patients with non-stricturing/non-penetrating behavior exhibited a modification of this characteristic to a more aggressive behavior. However, other studies have reported that 31% to 60% of patients exhibit a disease progression to a more severe behavior[32-34]. Indeed, after 40 years, most patients experience complications and are classified as having a penetrating, or less often, a stricturing disease[16]. These differences might reflect the short follow-up period used in the present study. Additionally, it has been reported that colonic disease remains uncomplicated or inflammatory for many years[24], so predominant colonic involvement found in our study might play a role in this progression. Another factor potentially explaining this difference is that the patients at this institution were aggressively treated upon diagnosis with an "accelerated step-up approach", involving the initiation of biological therapy a median of one year after diagnosis for patients diagnosed since 2010; thus, patients might have a lower probability to exhibit a change in behavior[35,36].

During the IBD course, 12 patients (2.3%) with an initial diagnosis of UC developed perianal fistulas or showed ileal involvement, changing their diagnosis to CD, as confirmed through histological and image analyses. Previous studies have described a 5%-10% change in diagnosis after 25 years of the disease course[37]. This finding might reflect the short follow-up period in the present study.

The analysis of IBD treatment revealed that mesalamine was the most used drug in UC treatment (98%), whereas corticosteroids, mesalamine and immunosuppressive agents were used at equal frequencies (67%-68%) in CD treatment. In the present study, despite the frequent use of mesalamine for patients with CD, the use of this agent in CD is controversial. Indeed, the European Crohn's and Colitis Organization Consensus recently stated that oral aminosalicylates are not recommended for the treatment of mild to moderate CD[38]. However, both the American and British National Gastroenterology Associations recommend the use of high-dose 5-aminosalicylic acid as the first-line treatment of mild ileal, ileocolonic or colonic CD[38]. Because we had a high percentage of patients with colonic involvement, treatment with mesalamine could have had some implications because the action of mesalamine is predominantly topical at the site of inflammation, particularly within the colon[38]. However, many of these patients received mesalamine, regardless of the severity of the disease, prior to evaluation at this institution, possibly resulting from misinformation regarding the role of mesalamine in CD. On the other hand, according to the latest clinical guidelines, the use of immunosuppressive and biological therapies is significantly higher in CD patients compared with UC patients, as observed in the present study. In the series examined in the present study, the use of infliximab in UC treatment (7%) was similar to that reported in other countries[31,39,40]; however, the use of this drug in CD treatment (34%) was considerably higher than that detailed in Saudi Arabia, Israel and some European countries, which report frequencies between 2% and 10%[31,40]. Similarly, the use of immunomodulators was considerably higher in both groups compared with that observed in some European countries[31]. This discrepancy reflects the type of center where the present study was conducted, *i.e.*, a tertiary center that treats patients with more complex diseases. Unfortunately, the use of adalimumab and certolizumab pegol was extremely low in the cohort examined in the present study, reflecting the low coverage of these therapies by insurance companies. In addition, vedolizumab is still not available for use in Chile.

The investigation of the use of surgery for IBD treatment revealed that 38% of the CD patients required surgery for either intestinal resection or perianal disease. The frequency of intestinal resection was significantly higher in CD patients (25%) compared with that of colectomy in UC patients (5%), a result consistent with the findings reported by Niewiadomski *et al*[41], who showed that the risk of intestinal resection in CD was 13% after one year and 26% after five years. However, the colectomy rates in UC obtained in this study were 2% and 13% after one and five years, respectively. Notably, the early use of immunomodulators and biological therapies during the disease course could reduce the risk of surgery[24,42], particularly for those patients who achieve mucosal healing[43].

Relatively few data are available regarding the hospitalization rates in population-based cohorts[44,45]. The CD patients in the cohort examined in the present study had significantly more hospitalizations than the UC patients; however, higher percentages of patients belonging to both groups in this cohort were affected compared with the frequencies obtained in previous studies[41,46]. Nevertheless, it has previously been reported that more than one-third of UC patients require hospitalization within one year after diagnosis in the biological era[44]. Among the UC patients examined

in the present study, 35% required hospitalization at some point during the disease course. The disease extent at diagnosis and the need for steroids and anti-TNF therapy were associated with the risk of UC-related hospitalization[44]. For CD, a 52.7% cumulative risk of hospitalization within ten years of diagnosis has previously been described[45]. Among the CD patients in the present study, 55% required hospitalization at some point during the disease course.

The analysis of our data concerning IBD and *C. difficile* infection demonstrated that this bacterium was equally prevalent in patients with UC and patients with CD (5%). Notably, it is difficult to clinically distinguish between *C. difficile* infections and IBD flare-ups because both pathologies have similar presentations, *i.e.*, diarrhea and abdominal pain. Indeed, C. *difficile* might mimic or even trigger an IBD flare-up, and screening is therefore recommended at every flare-up experienced by these patients[47]. Because IBD patients with concomitant *C. difficile* infections have been associated with longer hospital stays, colectomy and even higher mortality, the diagnosis of this bacterial infection is important[48]. The data obtained in the present study differ from those detailed in previous publications, which reported that UC patients exhibit increased susceptibility to *C. difficile* compared with CD patients[48-50]; however, previous studies have reported that one of the major risk factors for *C. difficile* infection in patients with IBD is colonic IBD[51], and 44% of the patients with CD in the present study showed colonic involvement. Importantly, only patients with moderate-to-severe activity were examined for *C. difficile* infections, and hence, these data might be underestimated because patients with mild activity were not examined.

In conclusion, IBD has continued to increase in the present cohort, slowly approaching the levels reported in developed countries. The association of this institution with a multidisciplinary IBD program has improved the characterization of these patients and had therefore improved management options.

Limitations

The present study was conducted in a private single tertiary center, which may have resulted in bias because many of the patients received consultations for second opinions. Some of these individuals were inadequately treated, whereas others are refractory to therapy; therefore, these patients could represent more complex cases. In addition, more drugs are available for treatment in our center compared with those available at the public hospitals in Chile. In addition, the presented findings were obtained retrospectively, implying a selection bias. Nevertheless, we collected data from a considerable number of patients diagnosed with IBD, enabling a demographic and clinical characterization of these individuals. Unfortunately, in the present study, we were unable to determine incidence or prevalence rates because we did not receive patients from a determinate geographic area.

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COMMENTS

Background

Several studies have reported that the incidence of inflammatory bowel disease (IBD) has increased over the past several decades, even in countries where the frequency of this disease is low. Industrialization, increased physician awareness, advancements in diagnostic methods and greater access to medical services are factors that might explain this rise.

Research frontiers

Although few epidemiological studies have been conducted in Latin America, these studies have also described an increased incidence of IBD. The incidence and prevalence of IBD in Chile are unknown; however, increases in the numbers of Crohn's disease (CD) and ulcerative colitis (UC) cases have been suggested. The research goal of this study was to actualize previously published data to better demographically and clinically characterize IBD in patients from Chile.

Innovations and breakthroughs

The present study represents the largest series of IBD patients reported in Chile and even in South America. These data demonstrated an increase in the number of IBD cases.

Applications

The data used in this study not only enable the characterization of patients locally but also facilitate the comparison of these individuals with those included in other studies conducted worldwide. The characterization of these patients enabled treatment optimization, thereby improving patient quality of life.

Terminology

IBD includes a spectrum of typically progressive chronic diseases, including CD, UC and unclassified colitis. Although IBD mortality is low, the onset of this disease during early adulthood and its chronicity as a lifelong disease lead to a significant decline in the quality of life of IBD patients and an increase in the burden on the healthcare system due to high treatment costs.

Peer-review

The paper is good written, an intersting paper regarding IBD in developing country with different results about disease distrubution, severity and treatment.

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ORIGINAL ARTICLE

Observational Study

Primary biliary cirrhosis degree assessment by acoustic radiation force impulse imaging and hepatic fibrosis indicators

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Author contributions: Zhang FC and Hu RF designed the research; Zhu T, Tong L, Zhang QQ, and Zhang HC performed the research; Hu RF contributed new reagents/analytic tools; Zhang HC and Zhu T analyzed data; and Tong L and Zhang QQ wrote the paper.

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Abstract

AIM: To evaluate the assessment of primary biliary cirrhosis degree by acoustic radiation force impulse imaging (ARFI) and hepatic fibrosis indicators.

METHODS: One hundred and twenty patients who developed liver cirrhosis secondary to primary biliary cirrhosis were selected as the observation group, with the degree of patient liver cirrhosis graded by Child-Pugh (CP) score. Sixty healthy individuals were selected as the control group. The four indicators of hepatic fibrosis were detected in all research objects, including hyaluronic acid (HA), laminin (LN), type Ⅲ collagen (PC Ⅲ), and type Ⅳ collagen (Ⅳ-C). The liver parenchyma hardness value (LS) was then measured by ARFI technique. LS and the four indicators of liver fibrosis (HA, LN, PC Ⅲ, and Ⅳ-C) were observed in different grade CP scores. The diagnostic value of LS and the four indicators of liver fibrosis in determining liver cirrhosis degree with PBC, whether used alone or in combination, were analyzed by receiver operating characteristic (ROC) curve.

RESULTS: LS and the four indicators of liver fibrosis within the three classes (A, B, and C) of CP scores in the observation group were higher than in the control

group, with C class > B class > A class; the differences were statistically significant (P < 0.01). Although AUC values of LS within the three classes of CP scores were higher than in the four indicators of liver fibrosis, sensitivity and specificity were unstable. The ROC curves of LS combined with the four indicators of liver fibrosis revealed that: AUC and sensitivity in all indicators combined in the A class of CP score were higher than in LS alone, albeit with slightly decreased specificity; AUC and specificity in all indicators combined in the B class of CP score were higher than in LS alone, with unchanged sensitivity; AUC values (0.967), sensitivity (97.4%), and specificity (90%) of all indicators combined in the C class of CP score were higher than in LS alone (0.936, 92.1%, 83.3%).

CONCLUSION: The diagnostic value of PBC cirrhosis degree in liver cirrhosis degree assessment by ARFI combined with the four indicators of serum liver fibrosis is of satisfactory effectiveness and has important clinical application value.

Key words: Acoustic radiation force imaging technology; Hepatic fibrosis index; Primary biliary cirrhosis; Diagnostic value

Core tip: One hundred and twenty patients who had developed liver cirrhosis from primary biliary cirrhosis were assessed by ARFI imaging and hepatic fibrosis index alongside sixty healthy individuals. The ROC curves of LS combined with four liver fibrosis indexes showed that the AUC values (0.967), sensitivity (97.4%), and specificity (90%) of all indexes combined in the C grade of CP score were higher than in those of LS alone (0.936, 92.1%, and 83.3%). The diagnostic value of PBC cirrhosis degree in liver cirrhosis degree assessment by ARFI combined with the four indicators of serum liver fibrosis is of satisfactory effectiveness and has important clinical application value.

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INTRODUCTION

Primary biliary cirrhosis (PBC) is a chronic cholestatic disease[1] that can develop into liver fibrosis, cirrhosis[2-4], and even lead to liver failure[5]. When a patient is already in the liver cirrhosis stage, accurate diagnosis, and assessment of the extent of liver cirrhosis is vital to the diagnosis, treatment, and prognosis of the disease[6]. Therefore, exploring a high value examination method to diagnose liver cirrhosis is very significant[7-9]. It has been reported that liver cirrhosis can be divided into three classes according to the Child-Pugh (CP) scoring criteria, and the accuracy of their assessment methods have been demonstrated[10-13]. Although liver biopsy is still currently the preferred diagnostic method for cirrhosis, the resulting trauma to the patient's body leads to low acceptance[14-17]. Serum fibrosis indicators are a non-invasive examination method of cirrhosis diagnosis with a wide range of applications[18], however its accuracy in the assessment of cirrhosis degree remains to be studied[19]. Acoustic radiation force impulse imaging (ARFI) is a new ultrasound elastography technique[20] that can detect the hardness of the liver parenchyma for liver disease accurate assessment, and is non-invasive, simple, repeatable[21-23], and it can effectively compensate for the lack of liver biopsy and serum liver fibrosis markers. ARFI technology in China remains at the clinical development phase[24-26]. However, comparative studies of ARFI technology and other methods to assess the degree of liver cirrhosis and joint applications are few[27-30]. This study intends to use the CP score as a grading standard, as well as to observe the comparison of ARFI technology measured serum fibrosis markers alone and in combination with diagnostic accuracy to find a more satisfactory diagnostic method for PBC, with the aim of providing a theoretical basis for the clinical diagnosis and treatment of liver cirrhosis.

MATERIALS AND METHODS

General information

From January 2014 to September 2015, 120 patients with primary cholestatic cirrhosis that had developed to the stage of cirrhosis and were admitted to Huashan Hospital (Baoshan Branch Affiliated to Fudan University, Shanghai, China) were selected as the observation group. The patients consisted of 35 males and 85 females, with an average age of 56.33 ± 7.42 years. Patients were divided into different groups according to Child-Pugh score as follows: grade A, 39 cases; grade B, 43 cases; and grade C, 38 cases. Meanwhile, 60 healthy subjects were chosen as the control group, and consisted of 24 males and 36 females, with an average age of 54.27 ± 8.31 years. General information on the differences between these two groups was not statistically significant (P > 0.05). This study was approved by the ethics committee.

Diagnostic criteria

The degree of liver cirrhosis in patients was diagnosed based on symptoms, signs, CT, MRI, biochemical examination, and liver biopsy results.

Table 1 Child-Pugh scoring criteria

Indicator	Score		
	1 point	2 point	3 point
Hepatic encephalopathy (grade)	None	Slight	Occasional drowsiness
Ascites	None	Small amount of diuretics can be controlled	Numerous
Total bilirubin (μmol/L)	< 34	34-51	> 51
Albumin (g/L)	> 35	28-35	< 28
Prolonged prothrombin time(s)	< 4	4-6	> 6

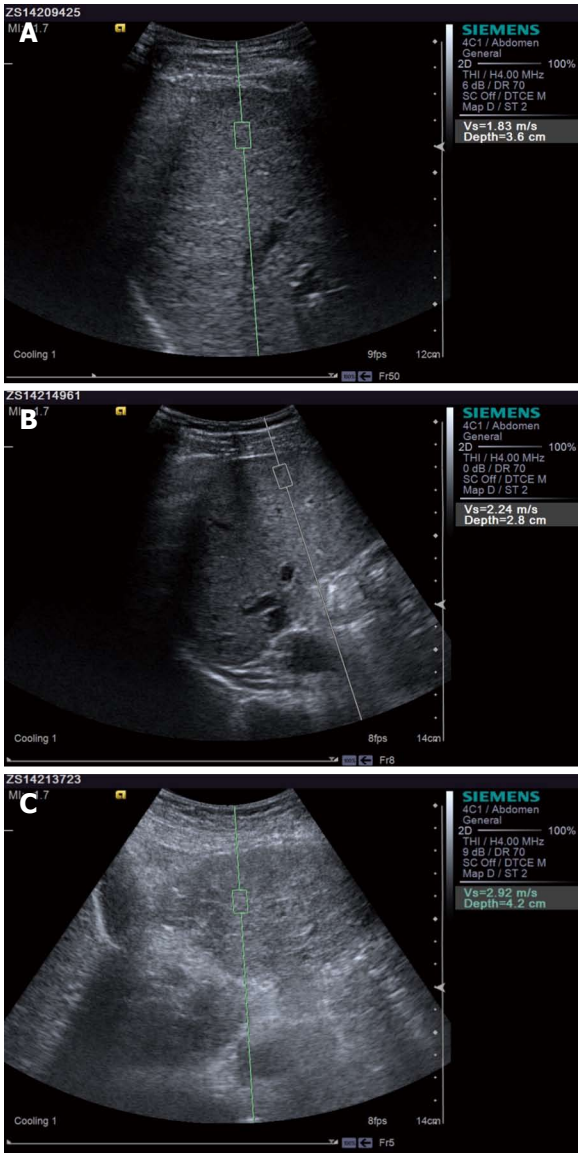

Figure 1 Observation group CP scores in the three classes. A: CP score patients with cirrhosis (Vs = 1.83 m/s); B: CP score patients with cirrhosis (Vs = 2.24 m/s); C: CP score patients with cirrhosis (Vs = 2.92 m/s).

Inclusion criteria

(1) Diagnosed with PBC that has developed to liver cirrhosis; (2) healthy subjects with no hepatobiliary diseases; (3) independent and able to cooperate with the test; and (4) provided written informed consent.

Exclusion criteria

(1) Patients with liver cancer or heart, lung, or other vital organs diseases; (2) disturbance of consciousness or mental illness; and (3) patients who provided written informed consent, but failed to cooperate with the test.

Child-Pugh scoring criteria

Patients were scored according to hepatic encephalopathy, peritoneal effusion, total bilirubin and albumin content, prolonged prothrombin time, and other conditions. Child-Pugh classification criteria (Table 1): class A, 5-6 points; class B, 7-9 points; and class C, ≥ 10 points.

Research methods

Liver fibrosis index detection: (1) After fasting, 5 ml of morning blood samples were collected from patients and kept at room temperature for approximately 30 min; (2) serum was separated and stored at -70 ℃; and (3) four indexes of liver fibrosis were determined using fluorescence immunoassay: hyaluronic acid (HA), laminin (LN), procollagen Ⅲ (PC Ⅲ), and collagen Ⅳ (Ⅳ-C).

ARFI detection: Siemens ACUSON S2000 color ultrasound diagnostic apparatus was used to conduct ARFI detection. (1) After fasting, the patient was placed on the left lateral position with the right hand on the head, and the right lobe of the liver tissue was detected; (2) elastic sampling frame was perpendicular to the surface of the liver, with a depth of approximately 2-5 cm while avoiding the surrounding blood vessels, and the patient was asked to hold their breath; and (3) the update button was pressed, a high-strength low-frequency pulse was launched, and the transverse shear wave velocity (Vs) was received. Units were in m/s and the value was recorded. Measurements were repeated 10 times and Vs were averaged to determine liver parenchyma hardness LS value.

Statistical analysis

SPSS 17.0 statistical software was used for all data results. LS value and the four indicators of liver fibrosis were measurement data presented as mean ± SD, with groups compared using two independent samples *t*-test. To evaluate the diagnostic value of LS value and the four serum indicators for liver fibrosis detected by ARFI (HA, LN, PCⅢ, and Ⅳ-C) for PBC, receiver operating characteristic (ROC) curve analysis with the area under the ROC curve (AUC), sensitivity and specificity representations were used. $P < 0.05$ was

Table 2 Test results of two groups of indicators (mean ± SD)

Item	Control group (n = 60)	Observation group		
		A class (n = 39)	B class (n = 43)	C class (n = 38)
LS value (m/s)	1.03 ± 0.03	1.90 ± 0.07[a]	2.31 ± 0.02[a]	2.92 ± 0.17[a]
HA (ng/mL)	54.96 ± 21.13	431.01 ± 118.04[a]	619.03 ± 164.28[a]	857.13 ± 192.05[a]
LN (ng/mL)	79.11 ± 15.37	116.14 ± 18.77[a]	153.42 ± 36.25[a]	211.09 ± 30.18[a]
PCⅢ (ng/mL)	89.91 ± 18.76	142.51 ± 30.07[a]	227.93 ± 69.11[a]	367.39 ± 99.21[a]
Ⅳ-C (ng/mL)	51.32 ± 9.27	104.58 ± 42.17[a]	168.99 ± 32.14[a]	193.36 ± 30.22[a]

[a]P < 0.01 *vs* the control group.

Table 3 Receiver operating characteristic curves results of different CP score classifications of liver cirrhosis with different indicators of diagnosis

Item	A Class			B Class			C Class		
	AUC	Sensitivity	Specificity	AUC	Sensitivity	Specificity	AUC	Sensitivity	Specificity
LS value	0.852	57.9%	93.3%	0.911	97.4%	75.0%	0.936	92.1%	83.3%
HA	0.694	97.4%	55.0%	0.852	97.4%	65.0%	0.888	63.2%	96.7%
LN	0.707	97.4%	43.3%	0.746	46.2%	96.7%	0.828	97.4%	58.3%
PCIII	0.741	57.9%	86.7%	0.823	53.8%	96.7%	0.871	86.8%	75.0%
IV-C	0.688	78.9%	56.7%	0.785	97.4%	51.7%	0.889	94.7%	73.3%

considered statistically significant.

RESULTS

Test result indicators in the two groups

CP scores, LS values, and the four serum indicators for liver fibrosis in the three classes (A, B, and C) of patients in the observation group were significantly higher than controls; the difference was statistically significant (P < 0.01). In the observation group, CP scores in the three classes of patients, LS values (Figure 1), and the four serum indicators for liver fibrosis revealed that class C > class B > class A; differences were statistically significant (P < 0.01), as shown in Table 2.

ROC curve analysis of LS value and the four indicators of serum liver fibrosis in the observation group

ROC curve analysis of LS values and the four diagnostic indicators of liver fibrosis of CP rates in different cirrhosis grades and each index of the AUC showed: grade C > grade B > grade A, as well as that the sensitivity and specificity were different (Table 3). Comparison of results of CP levels of LS values and the four indicators of liver fibrosis in the ROC curve are as follows:

In CP score grade A, LS values in the AUC and the specificity were high compared with serum liver fibrosis, albeit with lower sensitivity (Figure 2A).

In grade B, the AUC value of LS and specificity were high compared with HA and Ⅳ-C, but with lower sensitivity; AUC and sensitivity were high compared with LN and PCⅢ, but with lower specificity (Figure 2B).

In grade C, AUC values of LS, sensitivity, and specificity were high compared with PCⅢ; AUC and sensitivity were high compared with HA, but with lower specificity; AUC and specificity were high compared with LN and Ⅳ-C, but with lower sensitivity (Figure 2C).

ROC curve analysis of LS value in the observation group combined with the four indicators of serum liver fibrosis

LS values detected by ARFI in the observation group combined with the four indicators of serum liver fibrosis in the ROC curve show the following (Table 4): in each indicator of CP score grade A, the AUC and sensitivity were higher than the LS value detected by ARFI alone, although its specificity decreased slightly (Figure 3A); in CP score grade B, the AUC and sensitivity were higher than LS detected by ARFI alone, with sensitivity being constant (Figure 3B); in CP score grade C, the AUC, sensitivity, and specificity were higher than the LS values detected by ARFI alone (Figure 3C).

DISCUSSION

Cholestatic liver cirrhosis is a chronic liver disease with a long and gradual progression to liver cirrhosis[31-34]. An accurate assessment of early liver cirrhosis can effectively prevent further liver damage that can result in liver failure[35-37]; this has great significance for the diagnosis, treatment, and prognosis of chronic liver disease[38-40]. In this study, by comparing the diagnostic values of AFRI detected LS values and the four indicators (HA, LN, PCⅢ, and Ⅳ-C) of serum liver fibrosis alone or in combination, we aimed to accurately and effectively explore this examination

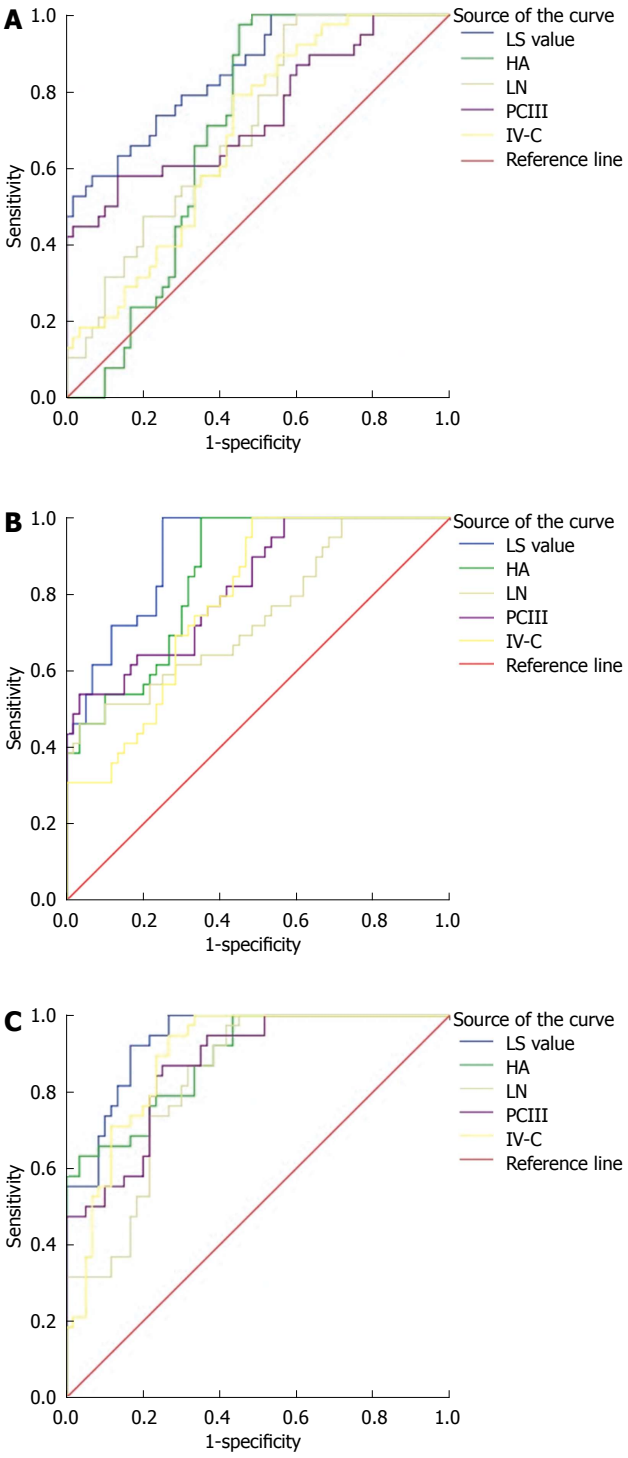

Figure 2 Comparison of the results of CP levels of LS values and the four indicators of liver fibrosis in the ROC curve. A: CP score for each indicator; B: CP score of the various indicators; C: CP score of the various indicators.

method for the assessment of cirrhosis degree.

LS values and the four indicators of serum liver fibrosis in observation and control groups

Liver stiffness increases as chronic liver disease develops to liver fibrosis and cirrhosis. In this study, the LS value results and four indicators of serum liver fibrosis in the observation group showed class C > class B > class A trends; the level of indicators were significantly higher. LS values and the four indicators of serum liver fibrosis of cirrhotic patients were higher than in the control group; this increased as liver cirrhosis degree increased. This also proves that ARFI-detected LS values and the four indicators of serum liver fibrosis can reflect changes in the degree of cirrhosis. Studies have reported[41] that AFRI-detected LS values increased as the degree of hepatic fibrosis increased; this can be widely used in patients with chronic liver disease. In recent years, this research has garnered more attention. The four serum fibrosis indicators for liver damage can be assessed via changes in each indicator, and thus can effectively diagnose cirrhosis. However, its detection accuracy for liver cirrhosis degree remains as yet unconfirmed[42].

Table 4 Receiver operating characteristic curve analysis for LS values combined with the four indicators of serum liver fibrosis

	LS value			Combination		
	AUC	Sensitivity	Specificity	AUC	Sensitivity	Specificity
A Class	0.852	57.9%	93.3%	0.881	68.4%	91.7%
B Class	0.911	97.4%	75.0%	0.973	97.4%	85.0%
C Class	0.936	92.1%	83.3%	0.967	97.4%	90.0%

ROC curve analysis of LS values and the four indicators of liver fibrosis

In the ROC curve analysis of LS value and the four indicators of liver fibrosis, we found the following: LS value and the four indicators of liver fibrosis in the AUC are present in class C > class B > class A trends, and that the diagnostic accuracy of each indicator can increase with increased liver cirrhosis degree (*i.e.*, each indicator can assess the degree of cirrhosis). While each indicator for the diagnostic value of different grades of liver cirrhosis are different, a comparison of results from the ROC curves show that the CP score of the three classes in the AUC were higher than in the four indicators of liver fibrosis, but that its sensitivity and specificity were unstable. CP score class A: LS values were higher than that of serum-specific liver fibrosis, but with lower sensitivity; CP score class B: LS values and specificity were higher than HA and Ⅳ-C, but with lower sensitivity (sensitivity was higher than LN and PCⅢ, but with lower specificity); CP score class C: LS values and sensitivity were higher than HA, but with lower specificity (specifically was higher than LN and Ⅳ-C, but with relatively lower sensitivity). The results show that the diagnostic value of LS values is high compared to the four indicators of liver fibrosis and that it has high diagnostic accuracy, although its diagnostic sensitivity and specificity is unstable. The sensitivity of the four indicators of liver fibrosis for the diagnosis of cirrhosis degree is strong, but its

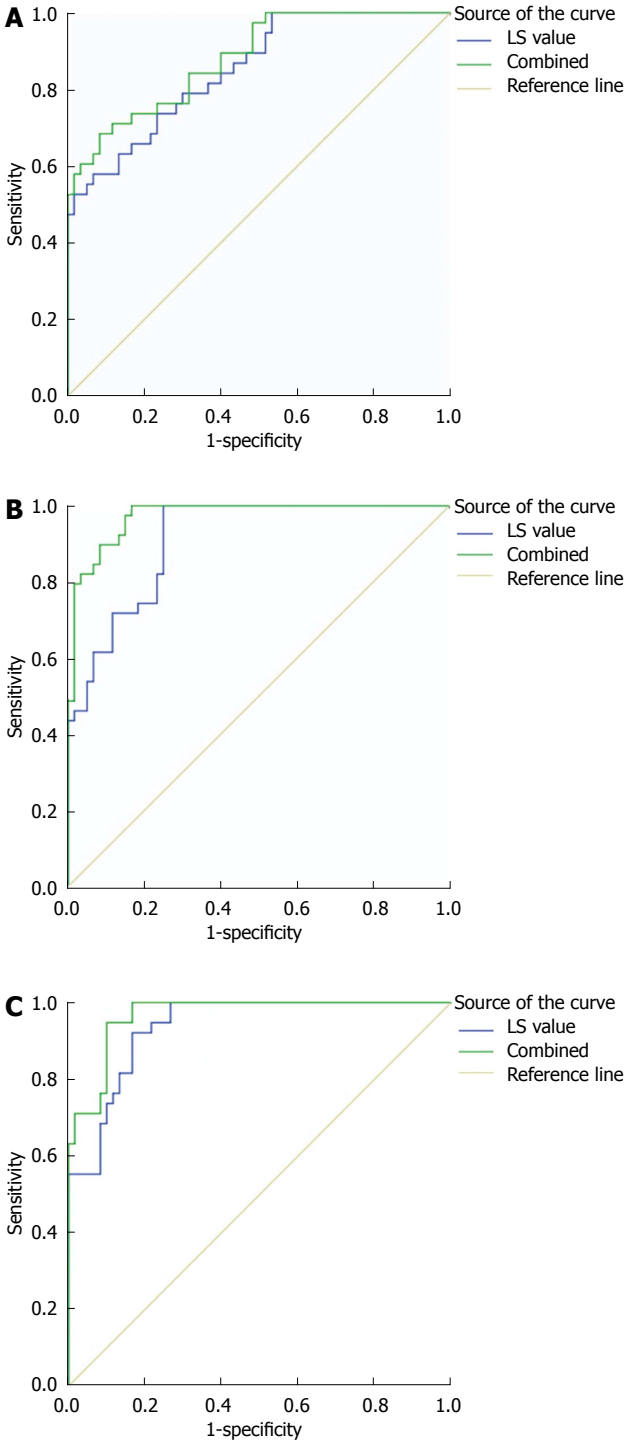

Figure 3 Receiver operating characteristic curve analysis for LS values combined with the four indicators of serum liver fibrosis. A: CP score of all indicators combined; B: CP score of all indicators combined; C: CP score of all indicators combined.

specificity and overall diagnostic value are insufficient. Detection of the four indicators of serum liver fibrosis can effectively diagnose cirrhosis, but lacks specificity in the accurate assessment of cirrhosis degree; thus, its technical support requires improvement[43]. The most commonly used method for the clinical diagnosis of cirrhosis is liver biopsy. However, due to its invasiveness, it has low acceptance limitations and causes more distress in clinical diagnosis and treatment to a certain extent[44-46]. On the other hand, ARFI ultrasound is a non-invasive detection technology. The degree of liver fibrosis can be determined by detecting LS value, which can compensate for the weakness of liver biopsy in detecting liver fibrosis[47].

ROC curve analysis of LS value combined with the four indicators of liver fibrosis

In the observation group, the results of the ROC curve analysis of LS value combined with the four indicators of serum liver fibrosis revealed that the CP score of the three classes combined with the diagnosis of AUC values were higher than ARFI-detected LS values alone, with sensitivity and specificity also improving. CP score class A: combined diagnosis sensitivity was higher than the LS value, albeit with slightly decreased specificity; CP score class B: the combined diagnostic specificity value was higher than the LS value, but sensitivity remained unchanged and there was no reduction; CP score class C: combined diagnostic sensitivity and specificity values were higher than the LS value. These results show that combined diagnosis improves the diagnostic accuracy of single-use LS values, and that diagnostic sensitivity and specificity can be guaranteed. The combined diagnostic value of LS values is high compared to the four indicators of liver fibrosis. It also proves that the LS value combined with the four indicators of serum liver fibrosis in the diagnosis of cirrhosis degree is higher than the diagnostic value of each indicator alone.

Limitation and prospects

Requirements for AFRI examination in patients were stringent. This may be due to insufficient coordination between doctors and patients, which affects the accuracy of the examination[48-50]. The detection operation for the four indicators of serum liver fibrosis is relatively simple, but also has its own shortcomings. Combined diagnosis can therefore play a complementary role and help improve diagnostic accuracy. Furthermore, LS values and indicators of liver fibrosis by way of motion detection can assist doctors in understanding the condition of a patient's liver disease, which is of great significance in the diagnosis and prognosis of liver cirrhosis.

In summary, the clinical diagnostic value of AFRI-detected LS value for determining liver cirrhosis degree is high compared to the four indicators of serum liver fibrosis. The diagnostic value of two combined diagnostics was more satisfactory compared to the indicators alone. Thus, detection by AFRI technology combined with the four indicators of serum liver fibrosis may serve as a powerful tool for determining liver cirrhosis degree, which has important clinical value and is worthy of wide promotion.

COMMENTS

Background

Primary biliary cirrhosis (PBC) is a chronic cholestatic disease that may develop into liver fibrosis, cirrhosis, and even lead to liver failure. When the patient is already in the liver cirrhosis stage, the accurate diagnosis and assessment of the extent of liver cirrhosis is vital in the diagnosis, treatment, and prognosis of the disease. Therefore, exploring a high value examination method to diagnose liver cirrhosis is very significant.

Research frontiers

It has been reported that liver cirrhosis can be divided into three classes according to the Child-Pugh (CP) scoring criteria, and the accuracy of their assessment methods have been demonstrated. Although liver biopsy is still currently the preferred diagnostic method for cirrhosis, the resulting trauma to the patient's body leads to low acceptance. Serum fibrosis indicators are a non-invasive examination method of cirrhosis diagnosis with a wide range of applications; however its accuracy in the assessment of cirrhosis degree remains to be studied. Acoustic radiation force impulse imaging (ARFI) is a new ultrasound elastography technique that can detect the hardness of the liver parenchyma for liver disease accurate assessment, and is non-invasive, simple, repeatable, and can effectively compensate for the lack of liver biopsy and serum liver fibrosis markers.

Innovations and breakthroughs

The clinical diagnostic value of AFRI-detected LS value for determining the degree of liver cirrhosis is high compared to the four indicators of serum liver fibrosis (HA, LN, PCIII, and IV-C). The diagnostic value of two combined diagnostics was more satisfactory compared to the indicators alone. Thus, detection by AFRI technology combined with the four indicators of serum liver fibrosis may serve as a powerful tool for determining liver cirrhosis degree, which has important clinical value and is worthy of wide promotion.

Applications

The diagnostic value of cirrhosis degree with PBC through liver cirrhosis degree assessment by ARFI combined with the four indicators of serum liver fibrosis is more satisfactory compared to the indicators alone and has important clinical application value. Results have shown that the higher the LS value, the higher the degree of liver fibrosis. This also confirmed that the diagnostic value of LS value was higher than that of the four indicators of liver fibrosis and, despite high diagnostic accuracy, that the diagnostic sensitivity and specificity were not stable. Further, the diagnosis value of liver cirrhosis degree for LS value combined with the four serum liver fibrosis was higher than each index alone.

Peer-review

The diagnostic value of cirrhosis degree with PBC through liver cirrhosis degree assessment by ARFI combined with the four indicators of serum liver fibrosis is more satisfactory compared to the indicators alone and has important clinical application value. The combination of AFRI and serum liver fibrosis four indicators can be used as a powerful tool to evaluate the degree of cirrhosis. It has important clinical application value and is worthy of clinical application.

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META-ANALYSIS

Hepatitis C virus genotype 3: Meta-analysis on sustained virologic response rates with currently available treatment options

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Abstract

AIM: To address the therapeutic efficacy of various treatment regimens in genotype 3 selecting randomized clinical trials and prospective National Cohort Studies.

METHODS: (1) PEG-INF-based therapy including sofosbuvir (SOF) + RBV for 12 wk *vs* SOF + RBV 24 wk; (2) SOF + RBV therapy 12 wk/16 wk *vs* 24 wk; and (3) the role of RBV in SOF + daclatasvir (DCV) and SOF + ledipasvir (LDV) combinations. This meta-analysis provides robust information with the intention of addressing treatment strategy for hepatitis C virus genotype 3.

RESULTS: A combination treatment including SOF + RBV + PEG-IFN for 12 wk notes better SVR than with only SOF + RBV for 12 wk, although its association with more frequent adverse effects may be a limiting factor. Longer duration therapy with SOF + RBV (24 wk) has achieved higher SVR rates than shorter durations (12 or 16 wk). SOF + LDV are not an ideal treatment for genotype 3.

CONCLUSION: Lastly, SOF + DCV combination is probably the best oral therapy option and the addition of RBV does not appear to be needed to increase SVR rates substantially.

Key words: Hepatitis C; Genotype 3; Sofosbuvir; Daclatasvir; Ledipasvir

Core tip: The landscape of therapy for hepatitis C virus infection is changing rapidly. In genotype 3, the improvement in SVR rates has not been hugely spectacular, being considered the most difficult genotype to treat and representing a major challenge. The advent of direct acting antivirals has not solved all questions about the treatment, while challenges remain such as the use of RBV, the duration of PEG-IFN-free treatment and whether PEG-IFN still plays an important role. These questions are difficult to elucidate with the current data because of the small number of patients included in clinical trials (particularly, those with cirrhosis) and their different designs.

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INTRODUCTION

The landscape of therapy for hepatitis C virus (HCV) infection is changing rapidly[1]. Ideally, new drugs should be all-oral regimen (once-daily, single pill) with pangenotypic activity, and have short treatment course (no more than 12 wk), and with high sustained virological response (at least 90%-95%). A multitude of direct acting antivirals (DAAs) have been developed with or without pegylated interferon (PEG-IFN) and ribavirin (RBV)[2], and others are being tested in promising clinical trials[3]. In genotype 3, the improvement in SVR rates has not relatively suboptimal and is being considered the most difficult genotype to treat and thus representing a major challenge[4]. Unique clinical features of genotype 3 and possible reasons for suboptimal response are: (1) a close relationship with insulin resistance and disturbances in lipid metabolism[5]; and (2) fibrosis progression[6] and higher incidence of hepatocellular carcinoma[7].

The advent of DAAs has not solved all questions regarding the treatment in genotype 3, and with emerging new challenges such as RBV use[8], duration of PEG-IFN-free treatment and whether PEG-IFN still plays an important role[9]. These questions are difficult to elucidate with the current data because of the small number of patients included in clinical trials (particularly, those with cirrhosis) and their different designs. In fact, more valuable data have been derived from prospective observational studies (clinical practice), and beyond randomized clinical trials. In this study, we aimed to address key questions on treatment outcomes through a meta-analysis.

MATERIALS AND METHODS

Data sources and search

The search strategy was in accordance with the recommendations of meta-analysis of clinical trials and observational studies. We searched in MEDLINE, EMBASE and Cochrane Library databases (to November 2015), as well as abstracts published and presented at EASL and AASLD (to November 2015) to identify potentially relevant publications in English language. We included FDA-approved DAA therapies that included SVR as a primary end point. Search terms were: "hepatitis C", "genotype 3", "HCV treatment", "sofosbuvir", "ledipasvir", "daclatasvir", "ribavirin", "interferon". The preceding terms were combined with appropriate Boolean logic. Manual search of cited bibliographies was also performed. Duplicated publications were deleted. Two researchers independently performed the literature search and data abstraction with regard to the inclusion and exclusion criteria by reading titles and abstracts. When reading titles and abstracts did not allow identification of eligible studies, articles were read in full. Relevant reviews and letters to the editor were excluded from the analysis, but read in full to identify potential relevant original studies. Disagreements between two observers were resolved by discussion.

Study selection criteria and data extraction

We selected randomized clinical trials (preferably) and prospective National Cohort Studies in which therapies were administrated in different arms. Therefore, studies including only a combination testing different doses or being administrated to different subset of patients were excluded. Inclusion and exclusion criteria (studies involving genotypes other than 3) were defined prior to initiation of the literature search. Twelve studies were included and classified according to the aims (Figure 1). The following data were extracted: (1) Study: year of publication, number of patients, location, design; (2) Patients: stage of liver disease (cirrhosis or chronic hepatitis), previous HCV treatment (naïve or treatment-experienced); (3) HCV treatment regimen and duration; and (4) SVR rates.

Objectives

We aimed to address the therapeutic efficacy of various treatment regimens in genotype 3. Firstly, we compared a PEG-INF-based therapy including sofosbuvir (SOF) + RBV during 12 wk with SOF + RBV 24 wk. Secondly, we assessed the importance of extending the course of SOF + RBV therapy (12 wk/16 wk *vs* 24 wk). Thirdly, we analyzed the role of RBV in SOF + daclatasvir (DCV) and SOF + ledipasvir (LDV) combinations.

Statistical analysis

Statistical analysis was performed using the Meta-Disc

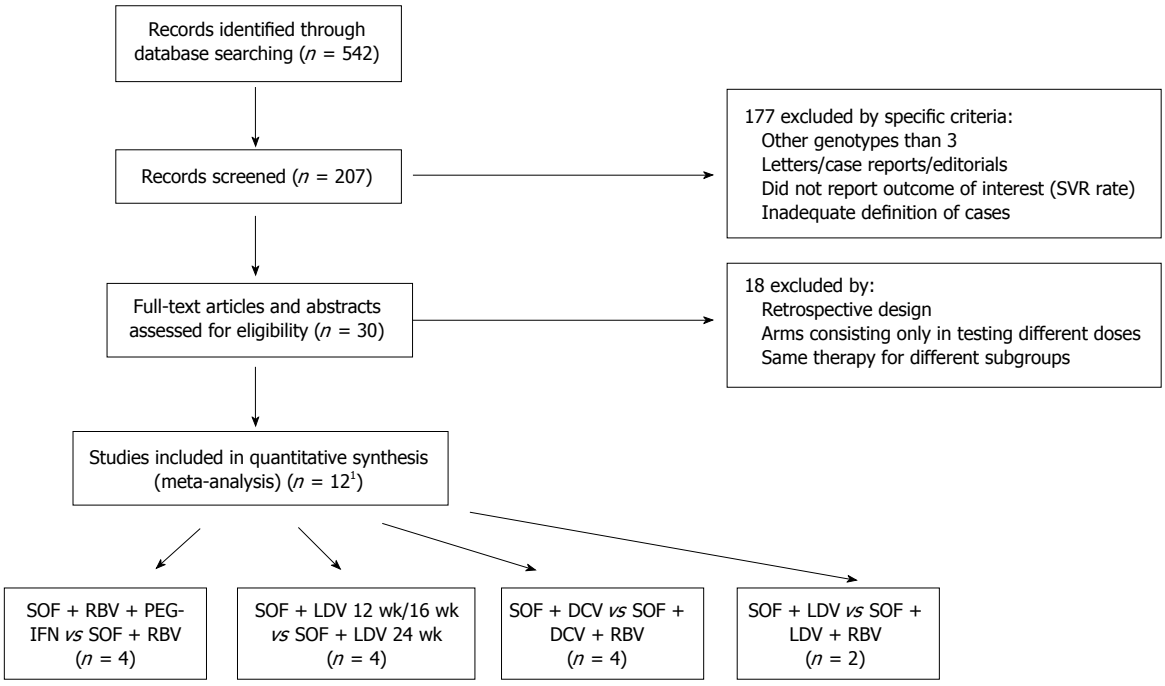

Figure 1 Flow chart of studies screened and included in meta-analysis. [1]Two studies included in two different sub-meta-analysis. SOF: Sofosbuvir; DCV: Daclatasvir; LDV: Ledipasvir; PEG-IFN: Polyethylene glycol interferon.

software 1.4[10], considering: (1) a summary of data from individual studies; (2) an investigation of the studies homogeneity, graphically and statistically; (3) calculation of clustered indexes; and (4) exploration of heterogeneity. Our assumption of heterogeneity was tested for each planned analysis using the Cochran-*Q* heterogeneity and I^2 statistics (low, moderate, and high heterogeneity according to I^2 values of 25%, 50%, and 75%, respectively)[11]. Random effects model using Der Simonian and Laird method and fixed effects model were used according to the presence of heterogeneity. To check for publication bias, we used the Begg and Egger tests. Only two-sided tests with a significance level of 0.05 were used. Confidence intervals (CIs) of individual studies were determined or approximated from the available data. Further, we assessed the quality of the studies using the "Quality Assessment of Diagnostic Accuracy Studies" (QUADAS) tool for observational studies ($\geqslant$ 10 were considered as high-quality studies[12]) and Jadad scale for randomized clinical trials ($\geqslant$ 3 were considered as high-quality ones[13]).

RESULTS

Comparison between INF-based and IFN-free regimens

We evaluated four studies that met the selection criteria and that were identified using the search strategy described. Studies characteristics are shown in Table 1. Pooled data included 807 patients. The meta-analysis demonstrated that triple therapy including SOF + RBV + PEG-IFN was able to achieve higher SVR rates (92.5%; 236/255) than SOF + RBV (75.2%; 415/552), using fixed effects model [OR = 3.51 (95%CI: 2.08-5.92)] (Figure 2A). We found neither heterogeneity between these studies [(Cochran-*Q* = 0.94; *df* = 3, *P* = 0.8157); inconsistency I^2 = 0%, and τ^2 = 0.0000)] nor publication bias [(Begg test: Kendall' s tau 1.70, *P* = 0.1); (Egger test: -1.14, *P* = 0.37)].

Course of SOF + RBV treatment

We included four studies involving 850 patients. The meta-analysis demonstrated that a 24 wk-course of SOF + RBV (85.5%; 501/586) combination was better than 12 wk-16 wk (70%; 185/264) in terms of SVR rates, using random effects model [OR = 3.51 (95%CI: 1.59-7.70)] (Figure 2B). We found a moderate heterogeneity between these studies [(Cochran-Q = 7.77, *df* = 3, *P* = 0.0511); inconsistency I^2 = 61%, and τ^2 = 0.3718], but no publication bias [(Begg test: Kendall's tau 0.34, *P* = 0.73); (Egger test: 0.81, *P* = 0.50)]. Three of these studies evaluated SVR rates according to the presence of cirrhosis. In non-cirrhotic patients, longer therapy of SOF + RBV (89.7%; 218/243) achieved higher SVR rates than shorter one (78.2%; 144/184) using random effects model (OR 2.44 (95%CI: 1.41-4.23). We did find a moderate heterogeneity between these studies [(Cochran-*Q* = 4.42; *df* = 2, *P* = 0.11); inconsistency I^2 = 55%, and τ^2 = 0.3987], with no publication bias. Similarly, this effect was observed in cirrhotic population (78.5%; 73/93 *vs* 55%; 38/69) using the random effects model [OR = 2.79 (95%CI: 1.34-5.78)].

Role of RBV in SOF + DCV and SOF + LDV combinations

Additionally, we assessed the role of adding RBV in

Table 1 Overall characteristics of studies included in meta-analysis

Ref.	Year	Patients characteristics	Study design	Outcome (SVR %)
Alqahtani *et al*[31]	2015	HCV mono-infected patients TARGET cohort Randomized by cirrhosis and previous treatment 50% Treatment naïve 51% Cirrhosis	a) SOF + RBV + PEG-IFN (n = 18) b) SOF + RBV (n = 133)	a) 89% b) 65%
Chulanov *et al*[32]	2014	HCV mono-infected patients Russian multicenter cohort Randomized by cirrhosis 100% Treatment naïve 18% Cirrhosis	a) SOF + RBV 16 wk (n = 30) b) SOF + RBV 24 wk (n = 31)	a) 87% b) 90%
Dalgard *et al*[33]	2015	HCV mono-infected patients Scandinavian cohort study 51% Treatment naïve 82% Cirrhosis	a) SOF + RBV + PEG-IFN 12 wk (n = 25) b) SOF + RBV 24 wk (n = 33)	a) 92% b) 79%
Foster *et al*[17](BOSON)	2015	HCV mono-infected patients Randomized study 51% Treatment naïve 31% Cirrhosis	a) SOF + RBV + PEG-IFN 12 wk (n = 181) b) SOF + RBV 16 wk (n = 181) c) SOF + RBV 24 wk (n = 182)	a) 93% b) 71% c) 84%
Foster *et al*[27]	2015	HCV mono-infected patients NHS England Early Access Program 100% Decompensated Cirrhosis	a) SOF + DCV 12 wk (n = 7) b) SOF + DCV + RBV 12 wk (n = 113) c) SOF + LDV 12 wk (n = 7) d) SOF + LDV + RBV 12 wk (n = 61)	a) 71% b) 81% c) 57% d) 72%
Gane *et al*[29] (ELECTRON-2)	2015	HCV mono-infected patients Randomized study 50% Treatment naïve 32% Cirrhosis	a) SOF + LDV 12 wk (n = 25) b) SOF + LDV + RBV 12 wk (n = 26) c) SOF + LDV + RBV 12 wk (n = 50)	a) 64% b) 100% c) 82%
Hezode *et al*[34]	2015	HCV mono-infected patients French Compassionate Use Program 27% Treatment naïve 94% Cirrhosis	a) SOF + DCV 12 wk (n = 26) b) SOF + DCV + RBV 12 wk (n = 4) c) SOF + DCV 24 wk (n = 35) d) SOF + DCV + RBV 24 wk (n = 13)	a) 85% b) 100% c) 91% d) 92%
Ingiliz *et al*[35]	2015	HCV-HIV co-infected patients German multicenter cohort study 50% Treatment naïve38% Cirrhosis	a) SOF + RBV + PEG-IFN 12 wk (n = 31) b) SOF + RBV 24 wk (n = 23)	a) 94% b) 91%
Sulkowski *et al*[22](PHOTON)	2014	HCV-HIV co-infected patients International multicenter cohort 25% Treatment naïve	a) SOF + RBV 12 wk (n = 42) b) SOF + RBV 24 wk (n = 123)	a) 67% b) 89%
Sulkowski *et al*[36]	2014	HCV mono-infected patients Randomized study 100% Treatment naïve 14% Cirrhosis	a) SOF + DCV 24 wk (n = 13) b) SOF + DCV + RBV 24 wk (n = 5)	a) 92% b) 80%
Welzel *et al*[28]	2015	HCV mono-infected patients European Compassionate Use Program 72% Cirrhosis	a) SOF + DCV 24 wk (n = 11) b) SOF + DCV + RBV 24 wk (n = 13)	a) 100% b) 85%
Zeuzem *et al*[37] (VALENCE)	2014	HCV mono-infected patients Randomized study 41% Treatment naïve 24% Cirrhosis	a) SOF + RBV 12 wk (n = 11) b) SOF + RBV 24 wk n = 250)	a) 27% b) 84%

HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; SOF: Sofosbuvir; DCV: Daclatasvir; LDV: Ledipasvir; PEG-IFN: Polyethylene glycol interferon.

IFN-free regimens. Four studies have evaluated this point regarding the combination treatment of SOF + DCV. Pooled data included 502 patients. The meta-analysis demonstrated that adding RBV was not essential to achieve optimal SVR rates (83%; 173/209 *vs* 86.3%; 253/293), using fixed effects model [OR = 1.09 (95%CI: 0.35-3.40)] (Figure 2C). We did not find heterogeneity between these studies [(Cochran-Q = 2.38; df = 3, P = 0.4981); inconsistency I^2 = 0%, and τ^2 = 0.0000], and did not seem to have publication bias. On the other hand, two studies have evaluated the role of adding RBV in SOF + LDV combination. Pooled data included 169 patients. The meta-analysis demonstrated that adding RBV was important to achieve better SVR rates (81%; 111/137 *vs* 62.5%; 20/32), using fixed effects model [OR = 3.30 (95%CI: 1.35-8.04)] (Figure 2D). We did not find heterogeneity between these studies [(Cochran-Q = 0.61, df = 1, P = 0.4335); inconsistency I^2 = 0%, and τ^2 = 0.0000], and no publication bias was found [(Begg test: Kendall's tau 0.01, P = 0.99)].

DISCUSSION

New challenges have emerged in the evolving era of HCV therapy, particularly with genotype 3, and these

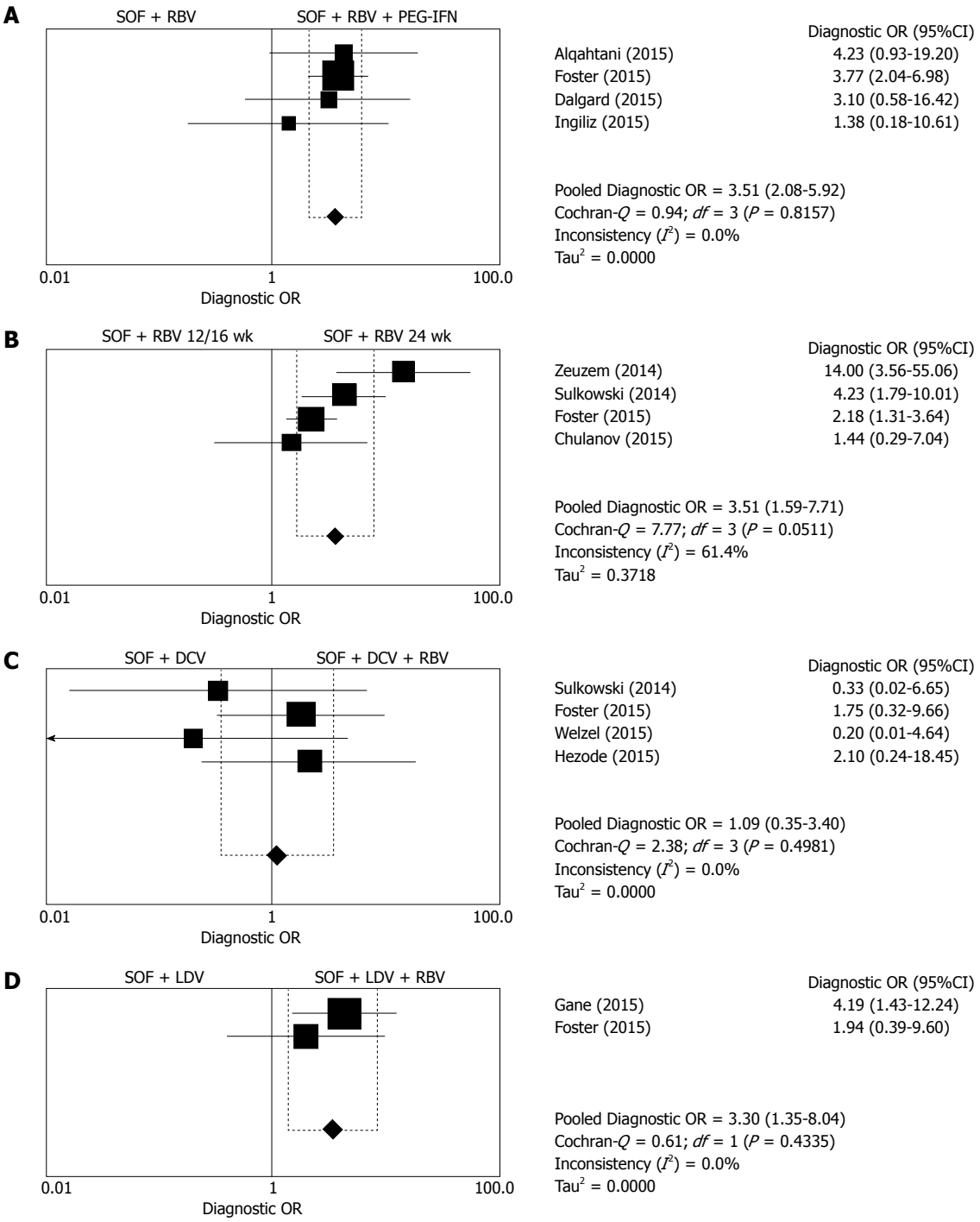

Figure 2 Odds ratio (95%CI) and Forest plot for SVR rates. A: SOF + RBV + PEG-IFN *vs* SOF + RBV combinations; B: SOF + RBV 12 wk/16 wk *vs* SOF + RBV 24 wk combinations; C: SOF + RBV 12 wk/16 wk *vs* SOF + RBV 24 wk combinations; D: SOF + LDV *vs* SOF + LDV + RBV combinations.

include the ongoing role of PEG-IFN, the addition of RBV and the adequate duration of the therapy[13]. The rapid development and use of DAAs in several heterogeneous studies including small number of patients has made robust guideline development and recommendation rather challenging. Thus, a meta-analysis is needed pooling all patients to address these questions.

In this new era, PEG-IFN is being abandoned as part of standard HCV therapy because of the association with serious adverse effects (and the parenteral administration)[14]. From now on, PEG-IFN will not be used for genotypes 1, 2 or 4 anymore. For genotype 3, there are only two DAAs (SOF and DCV) with a significant inhibitory activity *in vitro*[15]. In this context, PEG-IFN could potentially play a role in HCV treatment and could be the last such indication for its use. We demonstrated that the addition of PEG-IFN to SOF + RBV 12 wk was superior to only SOF + RBV combination (92% *vs* 75%, OR = 3.51). BOSON study

represents the main study evaluating this comparison, and it included nearly two hundred patients per arm[16]. Additionally, DCV has been evaluated in combination with PEG-IFN + RBV, although SVR rates were not higher than those patients treated with dual standard therapy (65% *vs* 59%)[17]. Both EASL and AASLD recommend SOF + RBV + PEG-IFN as a good alternative in non-cirrhotic and compensated-cirrhotic patients[18]. On the other hand, no data is available evaluating SOF + RBV + PEG-IFN *vs* SOF + DCV.

We analyzed the combination of SOF + RBV, in terms of duration of therapy. To date, this combination has been evaluated for 12, 16 and 24 wk duration. We compared SOF + RBV 12 wk/16 wk *vs* SOF + RBV 24 wk, and the latter achieved higher SVR rates (89% *vs* 70%, OR = 3.51). Furthermore, SOF + RBV 12 wk (56%) was associated with poorer SVR rates than dual standard therapy with PEG-IFN + RBV 24 wk (63%) in FISSION study[19], and showing similar results than POSITRON study (61%)[20]. Both studies demonstrated that SOF + RBV combination 12 wk was suboptimal, especially in the cirrhotic population. In FISSION study, a longer course of therapy (16 wk) with SOF + RBV showed better results than a shorter one (62% *vs* 30%)[21]. Overall SVR rates with SOF + RBV 12/16 wk were about 60%, which is considered suboptimal in the evolving era of hepatitis C therapy where response rates far below 90% are considered suboptimal. We included four studies that evaluated the course of 24 wk of SOF + RBV and noted an overall SVR rate around 90%. In addition, PHOTON study confirmed the extrapolation of these results in HIV-co-infected patients[22]. Taking into account all of these results, EASL and AASLD guidelines recommend extending SOF + RBV treatment to 24 wk (especially indicated in non-cirrhotic population).

In this meta-analysis, we demonstrated that SOF + LDV combination needs the addition of RBV to achieve optimal SVR rates in patients with genotype 3 (81% *vs* 62%, OR = 3.30). In contrast, RBV did not play any role in the combination of SOF + DCV because it did not improve SVR rates. DCV and LDV are HCV NS5A inhibitors[23], although DCV shows a pangenotypic activity[24] while LDV has a low activity in genotypes 2 and 3[25]. Currently, SOF + DCV combination is the first option to treat patients with genotype 3 in EASL guidelines, 12 wk in non-cirrhotic and 24 wk (with RBV) in cirrhotic patients. This recommendation is mainly based on ALLY-3 study in which SOF + DCV 12 wk achieved 97% and only 58% SVR in non-cirrhotic and cirrhotic population respectively[26]. The UK Early Access Program did not show any impact of adding RBV to SOF + DCV 24 wk in cirrhotic patients (70% *vs* 71%)[27], as well as the European Compassionate Use Program in patients at high risk of hepatic decompensation or death within 12 mo (100% *vs* 85%, *P* = NS)[28]. In a relatively small study, ELECTRON-2 trial, SOF + LDV for 12 wk achieved suboptimal SVR rates while the addition of RBV substantially increased it (100% in non-cirrhotic naïve patients, and 89% in non-cirrhotic and 73% in cirrhotic treatment-experienced patients)[29]. However, this trial should be interpreted with caution because it has very limited data from a phase Ⅱ single-center study and comprising a homogenous population which could limit the generalizability of the results. This, together with the high EC50 of LDV for genotype 3[30], has lead EASL and AASLD to not recommend SOF + LDV±RBV combination for genotype 3.

Recommendations made by EASL and AASLD guidelines were based on few data derived from randomized clinical trials and, due to the rapid and wide use in clinical practice, modified by prospective national cohorts. This meta-analysis provides solid and robust information to address several important questions, regarding the treatment of HCV genotype 3. First, combination including SOF + RBV + PEG-IFN shows better results than only SOF + RBV, although its association with adverse effects may limit the use (*i.e.,* cirrhotic population). Second, longer therapies including SOF + RBV (24 wk) have higher SVR rates than shorter ones (12 or 16 wk). Therefore, SOF + RBV for 24 wk are ideal. Third, SOF + LDV should not be used in genotype 3 and, if so, necessarily with RBV. Lastly, SOF + DCV combination is probably the best option and the addition of RBV does not appear to be needed to increase substantially the SVR rates.

COMMENTS

Background

The advent of direct acting antivirals has not solved all questions of successfully and effectively treating all hepatitis C virus (HCV) genotypes. Genotype 3, a common genotype globally, remains the last challenge.

Research frontiers

Nowadays, it remains unclear if Peg-IFN and RBV are still required to treat HCV genotype 3 effectively. The worldwide research is directed towards a more suitable combination of DAA.

Innovations and breakthroughs

In the present study, the authors investigated the SVR rates of different DAA combinations. This is the first report of a meta-analysis including sofosbuvir, daclatasvir, ledipasvir, peginterferon and ribavirin showing the eradication of the HCV infection.

Applications

The present report allows understanding the role of DAAs in the treatment of HCV genotype 3.

Peer-review

This systematic review and meta-analysis adds useful information for clinical practice and research.

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CASE REPORT

Laparoscopic restorative proctocolectomy with ileal pouch-anal anastomosis for Peutz-Jeghers syndrome with synchronous rectal cancer

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Author contributions: Wu B and Zhong ME designed the report; Zhong ME, Niu BZ and Ji WY collected the patient's clinical data and wrote the paper.

Institutional review board statement: This article was reviewed and approved by Peking Union Medical College Hospital Review Board.

Informed consent statement: The patient involved in this study gave her written informed consent prior to study enrollment.

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Abstract

We report on a patient diagnosed with Peutz-Jeghers syndrome (PJS) with synchronous rectal cancer who was treated with laparoscopic restorative proctocolectomy with ileal pouch-anal anastomosis (IPAA). PJS is an autosomal dominant syndrome characterized by multiple hamartomatous polyps in the gastrointestinal tract, mucocutaneous pigmentation, and increased risks of gastrointestinal and nongastrointestinal cancer. This report presents a patient with a 20-year history of intermittent bloody stool, mucocutaneous pigmentation and a family history of PJS, which together led to a diagnosis of PJS. Moreover, colonoscopy and biopsy revealed the presence of multiple serried giant pedunculated polyps and rectal adenocarcinoma. Currently, few options exist for the therapeutic management of PJS with synchronous rectal cancer. For this case, we adopted an unconventional surgical strategy and ultimately performed laparoscopic restorative proctocolectomy with IPAA. This procedure is widely considered to be the first-line treatment option for patients with ulcerative colitis or familial adenomatous polyposis. However, there are no previous reports of treating PJS patients with laparoscopic IPAA. Since the operation, the patient has experienced no further episodes of gastrointestinal bleeding and has demonstrated satisfactory bowel control. Laparoscopic restorative proctocolectomy with IPAA may be a safe and effective treatment for patients with PJS with synchronous rectal cancer.

Key words: Peutz-Jeghers syndrome; Laparoscopy; Ileal pouch-anal anastomosis; Restorative proctocolectomy; Multiple polyps in gastrointestinal tract

Core tip: Few options currently exist for the therapeutic management of Peutz-Jeghers syndrome with synchronous rectal cancer. Here, we present a patient diagnosed with Peutz-Jeghers syndrome with synchronous rectal cancer treated with laparoscopic restorative proctocolectomy with ileal pouch-anal anastomosis (IPAA). The patient has experienced no further episodes of gastrointestinal bleeding and has demonstrated satisfactory bowel control. To our knowledge, this is the first report on laparoscopic restorative proctocolectomy with IPAA performed for the treatment of Peutz-Jeghers syndrome with synchronous rectal cancer.

Zhong ME, Niu BZ, Ji WY, Wu B. Laparoscopic restorative proctocolectomy with ileal pouch-anal anastomosis for Peutz-Jeghers syndrome with synchronous rectal cancer. *World J Gastroenterol* 2016; 22(22): 5293-5296 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i22/5293.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i22.5293

INTRODUCTION

Peutz-Jeghers syndrome (PJS) is a rare, hereditary, autosomal dominant disorder characterized by multiple hamartomatous polyps in the gastrointestinal tract (mainly in the jejunoileum but also in the stomach and colon) and mucocutaneous pigmentation. It has also been reported to be associated with a high risk of malignancy, with a lifetime cancer risk of up to 93%, and it is caused by a germline mutation in the STK11 gene[1,2].

Few options currently exist for the therapeutic management of PJS with synchronous rectal cancer. Surgical strategies are commonly used to treat the sequelae of PJS, such as small bowel intussusception or neoplastic lesions, which may require enterectomy. To date, no studies have focused on PJS patients treated with laparoscopic restorative proctocolectomy with ileal pouch-anal anastomosis (IPAA).

Here, we present a patient diagnosed with PJS with synchronous rectal cancer that was treated with laparoscopic restorative proctocolectomy with IPAA.

CASE REPORT

A 42-year-old female patient presented with a 20-year history of intermittent bloody stool, poor appetite, abdominal pain and body-weight loss. Twenty years prior, she had experienced occasional abdominal pain, which subsided without treatment. Her family history confirmed that her father and children had similar symptoms. She had presented to our emergency department one month prior with acute gastrointestinal hemorrhaging.

Physical examination showed scattered, punctate, dark-blue areas of pigmentation on her lips (Figure 1) and the distal parts of her fingers. The patient was pale and extremely emaciated. Her body mass index (BMI) was 16. Rectal examination (in the knee-chest position) revealed the presence of a soft mass in the rectal lumen that was 6 cm from the anal verge, approximately 3 cm in diameter, located at nine o' clock, and difficult to move.

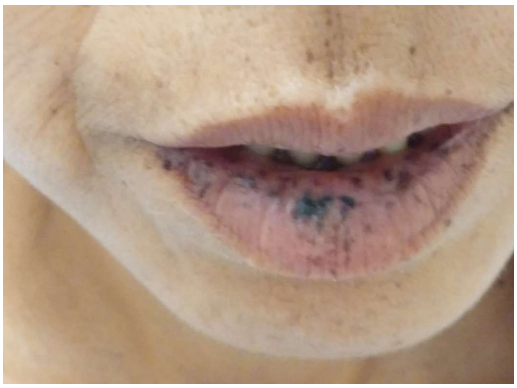

Figure 1 Scattered, punctate, dark blue macules on the mucosa of the lips.

Laboratory tests revealed that she had iron deficiency anemia (69 g/L hemoglobin). Her serum CA125 and serum CEA levels were within the normal limits. A computed tomography scan of the abdomen and pelvis revealed the presence of multiple polyps in the ileum and colorectum. Colonoscopy revealed the presence of multiple serried giant pedunculated polyps, with involvement of the rectum, sigmoid colon, descending colon, transverse colon and part of the ascending colon. In addition, a large cauliflower-like mass was identified at 7 cm from the anal verge (Figure 2). Biopsy of the tumor was performed, which revealed that the cauliflower-like mass was rectal adenocarcinoma (Figure 3A).

Finally, PJS with synchronous rectal adenocarcinoma was diagnosed. Surgical intervention consisting of laparoscopic restorative proctocolectomy and IPAA with a covering ileostomy was performed. During the operation, the patient was noted to have areas of intussusception of the small bowel secondary to the giant polyps. Several adenomatoid polyps were found at locations 30, 50 and 70 cm from the Treitz ligament, and consequently, enterotomy and polypectomy were performed.

The final pathological examination confirmed the diagnosis of moderately differentiated rectal adenocarcinoma (Figure 3B). Of the 37 lymph nodes examined, metastatic adenocarcinoma was detected in 3, and hamartomas with atypical hyperplasia were identified in the polyps, thereby confirming the diagnosis of PJS.

The patient underwent ileostomy closure 6 mo later. After 14 mo of follow-up, no further episodes of gastrointestinal bleeding occurred. This patient has demonstrated satisfactory bowel control to date. Her Wexner incontinence score is zero, indicating that she has no fecal incontinence. Her defecation frequency

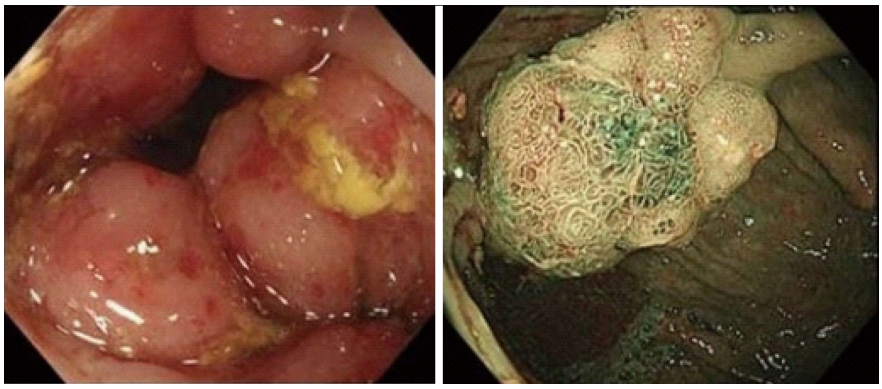

Figure 2 Colonoscopy revealed the presence of multiple polyps and a rectal cauliflower-like mass.

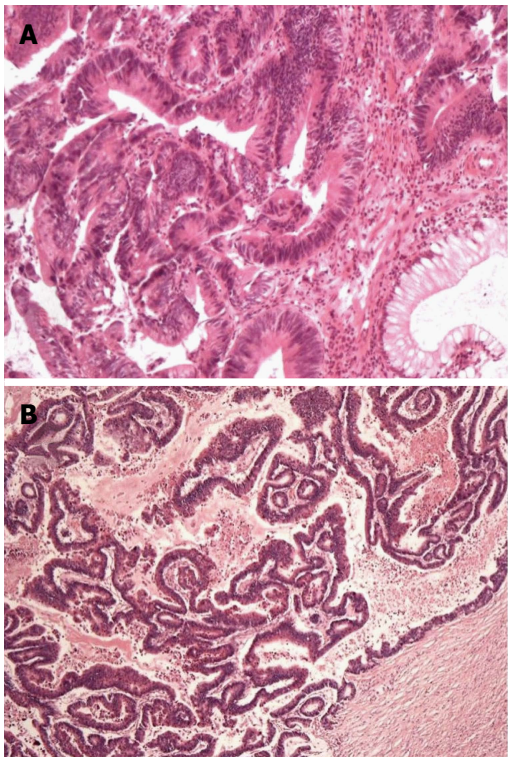

Figure 3 Biopsy revealed that the rectal mass was rectal adenocarcinoma (A) and postoperative pathological examination showed moderately differentiated rectal adenocarcinoma (B).

is 1-2/d. In addition, she is very satisfied with the cosmetic results.

One month ago, this patient underwent gastroenterography, and several polyps were found in the small bowel. As a result, double balloon enteroscopy (DBE) was performed. The ileal polyps were removed using an endoscopic snare.

DISCUSSION

PJS is an inherited, autosomal dominant disorder with variable inheritance and is characterized by hamartomatous polyps in the gastrointestinal tract, mainly in the small bowel, as well as pigmented mucocutaneous lesions. It is also characterized by increased risks for gastrointestinal and nongastrointestinal cancer. The prevalence of PJS is estimated to be between 1 in 50000 and 1 in 200000 individuals[2].

In this case, the patient had typical manifestations and a family history of PJS. Thus, it was not difficult to diagnose her. In addition, colonoscopy and biopsy revealed the presence of synchronous midrectal cancer.

Few options currently exist for the therapeutic management of PJS with synchronous rectal cancer. Surgical strategies are commonly used to treat the sequelae of PJS, such as small bowel intussusception or neoplastic lesions. Enterotomy and polypectomy or limited resection is considered to be the procedures of choice. Polypectomy using DBE is now recommended for small bowel polyps[3]. DBE is well recognized as a new enteroscopic method that allows for the examination and treatment of the jejunum and ileum in almost all patients. However, it cannot completely replace the surgical treatment of malignancies.

In the present case, we adopted an unconventional surgical strategy and performed laparoscopic restorative proctocolectomy and IPAA with a covering ileostomy.

Laparoscopic restorative proctocolectomy with IPAA is generally considered to be the first-line treatment option for patients with ulcerative colitis or familial adenomatous polyposis (FAP)[4]. However, no previous reports have focused on treating PJS patients with laparoscopic IPAA.

This report describes a patient who presented with severe gastrointestinal hemorrhage. Multiple polyps were found in her gastrointestinal tract, most of which were located in the colorectum and not the small bowl. Giant pedunculated polyps covered the patient's rectum, sigmoid colon, descending colon, transverse colon and part of the ascending colon, and surgery was necessary. In addition, colonoscopy and biopsy indicated the presence of a midrectal malignancy. Due to the locations of the giant polyps and the midrectal malignancy, restorative proctocolectomy with IPAA was considered. This procedure effectively minimizes

the risk of recurrence through the maximal removal of the involved bowel tissue while maintaining bowel continence. A large, retrospective cohort study from the Cleveland Clinic has shown that IPAA is a relatively safe and effective procedure with a low perioperative mortality rate of 0.1%[5]. Compared to conventional laparotomy, laparoscopic restorative proctocolectomy with IPAA is associated with less blood loss, fewer respiratory complications, a faster return of bowel function and a shorter hospital stay[6,7]. In addition, patients are satisfied with laparoscopic surgery[5], which significantly improves cosmesis and quality of life. In this case, the patient has not suffered from further episodes of gastrointestinal bleeding or fecal incontinence since the operation, and she is satisfied with the cosmetic results and the therapeutic effects.

We suggest that laparoscopic restorative proctocolectomy with IPAA may be a safe and effective treatment for PJS with synchronous rectal cancer in patients similar to the one presented in this report. However, this procedure cannot be applied to all patients with PJS. Polyps in the small intestine still require endoscopic polypectomy or resection of the involved regions of the small intestine.

COMMENTS

Case characteristics

A 42-year-old female patient presented with a 20-year history of intermittent bloody stool. Physical examination showed areas of pigmentation on the lips and extremities.

Clinical diagnosis

Peutz-Jeghers syndrome (PJS) with synchronous rectal cancer.

Differential diagnosis

Familial adenomatous polyposis.

Laboratory diagnosis

The hemoglobin level was 69 g/L, CEA, CA19-9 levels and metabolic panel and liver function test results were within the normal limits.

Imaging diagnosis

CT scan revealed the presence of multiple polyps in the ileum and colorectum.

Pathological diagnosis

Colonoscopy and biopsy revealed that the rectal mass was an adenoma with high-grade intraepithelial neoplastic changes. The final pathological examination confirmed the diagnosis of moderately differentiated rectal adenocarcinoma.

Treatment

The patient was treated with laparoscopic restorative proctocolectomy with ileal pouch-anal anastomosis (IPAA).

Experiences and lessons

The authors suggest that laparoscopic restorative proctocolectomy with IPAA may be a safe and effective treatment for treating patients with PJS with synchronous rectal cancer, with the advantage of minimal invasiveness.

Peer-review

In this manuscript, the authors reported a patient with PJS with synchronous rectal cancer who was treated with laparoscopic restorative proctocolectomy with ileal pouch-anal anastomosis. Overall, this case report is very interesting, and worthy to be published.

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CASE REPORT

Application of cystoscope in surgical treatment of hepatocellular carcinoma with portal vein tumor thrombus

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Author contributions: Cheng SQ designed the report and carried out the operation; Li N and Wei XB collected the clinic data and wrote the paper; Li N and Wei XB contributed equally to the article.

Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of Eastern Hepatobiliary Surgery Hospital.

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

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Abstract

Development of portal vein tumor thrombus deteriorates the prognosis of hepatocellular carcinoma, while surgical treatment can offer a promising prognosis for selected patients. However, the possibility of residual lesions in portal vein after conventional thrombectomy is a main risk factor leading to postoperative recurrence. Therefore, ensuring the complete removal of tumor thrombus during operation is critical to improve prognosis. For the first time, we report here one case of hepatocellular carcinoma with portal vein tumor thrombus in which cystoscope was successfully applied as a substitute of intravascular endoscope to visualize the cavity of the portal vein. The patient was a 61-year-old man with a 7-cm tumor in the right lobe of the liver, with tumor thrombus invading the right branch and adjacent to the conjunction of the portal vein. After removal of the tumor, the Olympus CYF-VA2 cystoscope was used to check the portal vein from the opening stump of the right branch of the portal vein. In this case, residual thrombus tissue was found near the opening stump and the conjunction of the portal vein. The residual lesion was carefully retrieved from the stump after retraction of the cystoscope. The procedure was repeated until no residual lesion was found. The whole duration time of thrombectomy was 22.5 (15 + 7.5) min. The patient was free from recurrence at 8 months after the procedure. Our work indicated that the cystoscope is a suitable substitute, with a proper size and function to check the portal vein system and ensure the curability of thrombectomy. Although well-designed clinic trails are still needed, this procedure may further improve the postoperative prognosis of hepatocellular carcinoma with portal vein tumor thrombus.

Key words: Hepatocellular carcinoma; Portal vein tumor thrombus; Surgical treatment; Thrombectomy; Cystoscope

Core tip: Inability to ensure the curability of the thrombectomy has been a main obstacle to improving postoperative prognosis of hepatocellular carcinoma with portal vein tumor thrombus, especially for cases with invasion in the main trunk of the portal vein. In this report, we firstly applied the cystoscope as an intravascular endoscope to investigate the cavity of the portal vein after primary tumor removal. The cystoscope offered a clear view of the portal vein cavity from the main trunk to the secondary branch, indicating its suitability as a substitute with a proper size and function to check the portal vein system.

Li N, Wei XB, Cheng SQ. Application of cystoscope in surgical treatment of hepatocellular carcinoma with portal vein tumor thrombus. *World J Gastroenterol* 2016; 22(22): 5297-5300 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i22/5297.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i22.5297

INTRODUCTION

Hepatocellular carcinoma (HCC) has a propensity to invade the intrahepatic vasculature, especially the portal vein system, leading to the formation of portal vein tumor thrombus (PVTT). PVTT is the most important significant factor for a poor prognosis, with a median survival of only 2.7 mo if patients are untreated[1]. Although sorafenib was recommended by the Barcelona Clinic Liver Cancer (BCLC) guideline as the only therapy for these patients, recent studies have demonstrated that surgical resection may offer a more promising prognosis for selected HCC patients with PVTT[2,3].

In the surgical operation, when the PVTT and tumor could not be resected *en-bloc*, thrombectomy was carried out after the removal of tumor. Theoretically, when thrombectomy was performed, squeezing or fragmenting the tumor thrombus could not be avoided, which would increase the risk of scattering tumor tissue within the portal vein cavity. What's more, there will be a possibility of residual PVTT tissues adhering to the inner wall of the portal vein even after careful extraction[4-6]. Those factors may lead to the early intrahepatic recurrence of tumor or PVTT[6]. Therefore, ensuring the complete removal of PVTT during operation is critical to improving the prognosis. With the development of endoscopy, it is theoretically ideal to achieve this goal by direct visual observation under intravascular endoscope. However, to the best of our knowledge, there is currently no angioscope specially designed for the portal vein system. Here, we describe one case of an HCC patient with PVTT in which the cystoscope was successfully applied as a substitute to the intravascular endoscope to visualize the cavity of the portal vein.

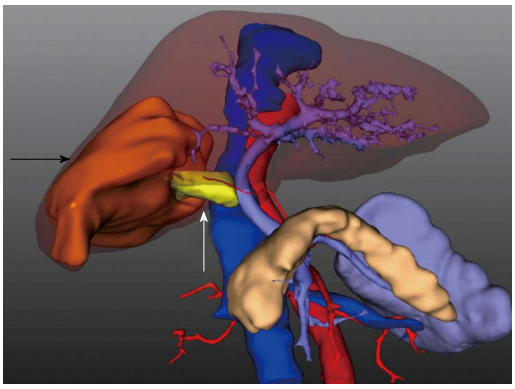

Figure 1 3D reconstruction of the tumor and portal vein tumor thrombus. A 7-cm hepatocellular carcinoma (white arrow) was located in segments Ⅴ, Ⅵ and Ⅶ. The tumor thrombus (black arrow) extended into the right branch and was adjacent to the conjunction of the portal vein.

CASE REPORT

This study was approved by our Institutional Review Board and written informed consent was obtained from the patient for this research. A 61-year-old man with hepatitis B virus infection presented to our department with a 7-cm HCC in the right lobe of the liver, with tumor thrombus that had invaded the right branch and was adjacent to the conjunction of the portal vein. Figure 1 shows a 3D reconstruction of the tumor and PVTT. The patient had Child-Pugh class A liver function and the other laboratory tests were normal. Intraoperative assessment confirmed the preoperative diagnosis. During operation, Pringle's maneuver was applied distal to the PVTT to occlude the blood inflow using a clamp/unclamp cycle of 15 min/5 min. According to characteristics of the tumor and the PVTT, a right semi-hepatectomy was carried out with a clamp crushing method.

After removal of the tumor, the Olympus CYF-VA2 cystoscope was used to check the portal vein. First, the streamlined tip was inserted into the opening stump on the right branch of the portal vein. The function of flush-and-suction was used to keep the field of view clear. In this case, scattered PVTT tissue was found near the opening stump. Further inspection revealed a residual lesion near the conjunction of the portal vein (Figure 2A and B). Then, the cystoscope was retracted from the stump and the residual PVTT was carefully retrieved using a clamp. After that, the portal vein cavity was reexamined meticulously from the main trunk to the left secondary branch by bending the flexible tip and drawing the insertion tube in and out. The procedure was repeated until no residual lesion was found (Figure 2C and D). Then, the stump was closed using a continuous suture. The whole duration time of the thrombectomy was 22.5 (15 + 7.5) min. The patient was discharged home without

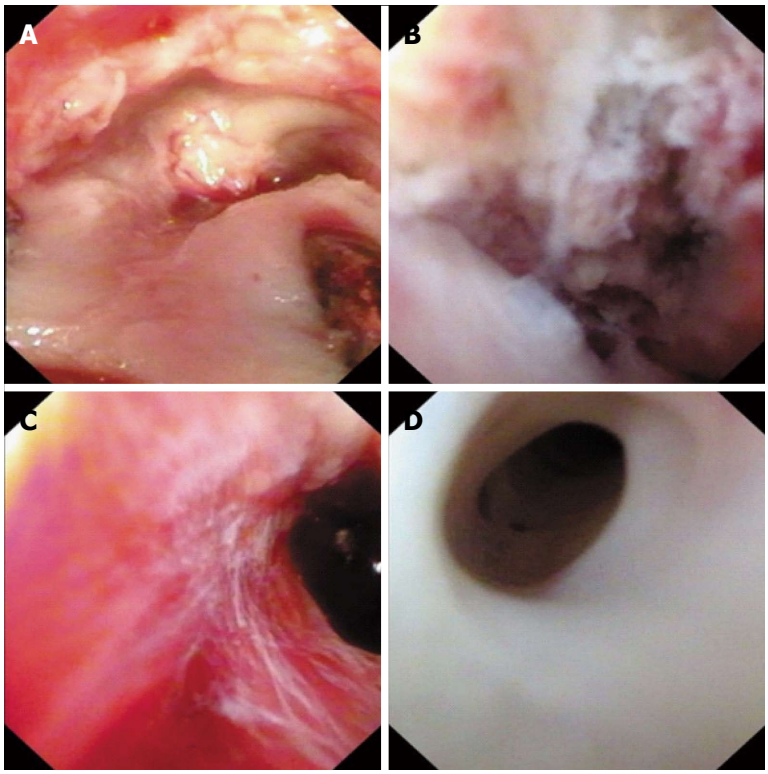

Figure 2 Endoscopic images of portal vein before and after thrombectomy. A: Before thrombectomy, endoscopy revealed scattered tissue of tumor thrombus near the opening stump; B: Residual tumor thrombus was adhered to the inner wall of the portal vein near the conjunction; C: After repeated retraction of the residual tumor thrombus, endoscopy revealed a clean inner wall of the portal vein with no macroscopic thrombus remaining; D: The left secondary branch of the portal vein was clean with no scattered thrombus.

complications on postoperative day 7 and was free from recurrence at 8 mo after the procedure when the last follow-up was attended.

DISCUSSION

Curative resection of tumor and complete removal of PVTT is essential to improve the oncological prognosis of HCC patients with PVTT. For PVTT confined to the ipsilateral branch of the portal vein, *en-bloc* resection of the ipsilateral portal vein branch containing the tumor thrombus has been recommended, whenever the liver remnant is sufficient[4,7]. However, for patients with PVTT extending to the main portal trunk, or patients with insufficient liver remnant after en-bloc resection, thrombectomy would be inevitably carried out after resection of the primary tumor. Patients who underwent thrombectomy have been reported to have a poor prognosis, with a 6-mo PVTT recurrence rate of 63.8% and the 1-year intrahepatic recurrence rate of 78.8%[4]. For these patients, residual or disseminated tumor thrombus in portal vein may be a significant risk factor leading to the high recurrence rate[6]. Therefore, it is crucial to eliminate the risk of residual thrombus while performing thrombectomy. Fortunately, the portal vein has no blood flow inside during the application of Pringle's maneuver, allowing the possibility of endoscopic inspection. In this case, the cystoscope we used could view the portal vein cavity clearly from the main trunk to the secondary branch, indicating it is a suitable substitute with a proper size and function to check the portal vein system. Despite the possibility that a microscopic lesion may still exist, this procedure theoretically eliminated the possibility of a residual and scattered macroscopic tumor thrombus in the portal vein and further ensured curability of the thrombectomy. This procedure may further improve the postoperative prognosis of HCC with PVTT**.** It will also be worthwhile to carry out a well-designed clinical trail to measure the significance of intravascular endoscopy to prove the postoperative prognosis of HCC with PVTT.

COMMENTS

Case characteristics

A 61-year-old man presented to our department with a 7-cm hepatocellular carcinoma (HCC) in the right lobe of the liver, with tumor thrombus invading the right branch and adjacent to the conjunction of the portal vein.

Treatment

Using a cystoscope to check the portal vein cavity after removal of the tumor in surgical treatment of HCC with portal vein tumor thrombus (PVTT).

Term explanation

PVTT means "portal vein tumor thrombus".

Experiences and lessons

The cystoscope we used could view the portal vein cavity clearly from the main trunk to the secondary branch, indicating it is a suitable substitute with a proper size and function to check the portal vein system in the surgical treatment of HCC with PVTT.

Peer-review

This is a novel idea to ensure the curability of hepatectomy for HCC with PVTT. The effectiveness of the treatment will require a well-designed clinical trail to further confirm.

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TOPIC HIGHLIGHT

2016 Colorectal Cancer: Global view

Fibroblasts, an inconspicuous but essential player in colon cancer development and progression

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Abstract

Tumor microenvironments have a crucial role in cancer initiation and progression, and share many molecular and pathological features with wound healing process. Unless treated, tumors, however, do not heal in contrast to wounds that heal within a limited time framework. Wounds heal in coordination of a myriad of types of cells, particularly endothelial cells, leukocytes, and fibroblasts. Similar sets of cells also contribute to cancer initiation and progression, and as a consequence, anti-cancer treatment strategies have been proposed and tested by targeting endothelial cells and/or leukocytes. Compared with endothelial cells and leukocytes, less attention has been paid to the roles of cancer-associated fibroblasts (CAFs), fibroblasts present in tumor tissues, because their heterogeneity hinders the elucidation on them at cellular and molecular levels. Here, we will discuss the origin of CAFs and their crucial roles in cancer initiation and progression, and the possibility to develop a novel type of anti-cancer treatment by manipulating the migration and functions of CAFs.

Key words: Angiogenesis; Drug resistance; Extracellular matrix; Immune evasion; Transforming growth factor-β; Invasion; Metastasis

Core tip: Tumor microenvironments have a crucial role in cancer initiation and progression, and consist of various types of cells, such as endothelial cells, leukocytes, and fibroblasts. Compared with endothelial cells and leukocytes, less attention has been paid to the roles of cancer-associated fibroblasts (CAFs), fibroblasts present in tumor tissues, because their heterogeneity hinders the elucidation on them at cellular and molecular levels. Here, we will discuss the origin of CAFs and their crucial roles in cancer initiation and progression, and the possibility to develop a novel type of anti-cancer treatment by manipulating the migration and functions of CAFs.

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INTRODUCTION

Dvorak proposed that tumors are wounds that do not heal[1], based on his discovery of vascular endothelial growth factor (VEGF), which is produced in wound healing sites as well as tumor sites[2] and can account for chronic hyperpermeability-mediated fibrin deposition in solid tumors and in early stages of wound healing[3]. Moreover, solid tumors and wound healing process share many pathological and molecular features.

Irrespective of the causes and the severities of wounds, healing proceeds to repair the structure and functions of injured organs and tissues, through a series of processes; hemostasis, humoral inflammation with microvascular permeability and extravascular clotting, cellular inflammation with inflammatory cell infiltration, angiogenesis, and generation of mature connective tissue stroma[4]. These steps proceed through the interaction between the parenchymal cells and the stroma, a complex mixture of inflammatory cells, matrix proteins, and tissue cells such as fibroblasts and endothelial cells. Injured organs and tissues are completely replaced by proliferating parenchymal cells in the case of acute and mild wound, but if not replenished completely, they are filled with connective tissue. Tumor cells, regardless of their site of origin, behave like parenchymal cells in normal tissues and proliferate by interacting with the stroma[4].

Fibroblasts are a major cell type within the stroma and contribute to tissue remodeling in development and tissue homeostasis, by providing structural scaffolding and growth regulatory mediators. Moreover, following tissue injury, fibroblasts exhibit an activated and contractile phenotype with enhanced expression of α-smooth muscle actin (α-SMA) and are referred to as myofibroblasts[5]. Myofibroblasts synthesize increased amount of various types of collagens and extracellular matrix proteins (ECM) to provide scaffold and to eventually aid in wound repair[5]. Furthermore, the cells are important sources of many growth factors and cytokines that regulate wound healing processes[6].

Primary normal fibroblasts isolated from various human tissues, can restrict the *in vitro* cell proliferation of various types of human cancer cell lines, under co-culture condition[7]. Indeed, if Nod-like receptor pyrin domain-containing protein 6 (NLRP6) is absent in fibroblasts within the stem cell niche in the colon, regeneration of the colonic mucosa and processes of epithelial proliferation and migration are impaired and consequently, colitis-associated tumorigenesis is accelerated in mice[8]. Thus, under normal conditions, fibroblasts work as a sentinel cell to maintain epithelial tissue homeostasis and to prevent initiation of tumorigenesis in colon, in a NLRP6-dependent manner.

Like fibroblasts in wound healing process, fibroblasts present in tumor tissues exhibit an activated and a myofibroblast-like phenotype with α-SMA expression and are referred as cancer-associated fibroblasts (CAFs)[9]. In contrast to fibroblasts in normal tissues, CAFs in most solid tumors are presumed to promote tumor development and progression by providing cancer cells with a myriad of growth factors[9,10]. However, in pancreatic ductal cancer, CAFs can deliver immune stimulating signals. As a result, depletion of CAFs induces immunosuppression with increased intra-tumoral regulatory T cells (Tregs) and eventually accelerates tumor progression with reduced survival[11]. Hence, it still remains elusive on the pathophysiological roles of CAFs in the development and progression of solid tumors.

We will herein discuss the pathophysiological roles of CAFs and their clinical relevance mainly in colorectal cancer (CRC), but will mention CAFs in other types of cancer if necessary.

CAFS IN COLITIS-ASSOCIATED COLON CARCINOGENESIS MODEL

Accumulating evidence indicates the crucial contribution of chronic inflammation to tumor development and progression[12]. Colitis-associated colon carcinogenesis (CAC) is one typical example of this pathological process. CAC frequently ensues from chronic intestinal inflammatory changes observed in patients with inflammatory bowel diseases such as ulcerative colitis (UC), particularly those with a long duration, extensive involvement, and severe inflammation[13]. Pathological features of UC include mucosal damage and ulceration with prominent leukocyte infiltration, and these changes involve rectum at first and extend proximally.

Oral administration of dextran sulfate sodium (DSS) solution to rodents can cause acute inflammatory reaction and ulceration in the entire colon, similar to that observed in human UC patients, and therefore, is widely used to reproduce human UC[14]. Moreover, repeated DSS ingestion alone can cause a small number of colon carcinomas in about a half of mice[15], suggesting that inflammatory response alone can cause colon carcinoma. The incidence of DSS-induced colon carcinogenesis is accelerated and increased by a prior administration of azoxymethane (AOM)[16], which can alone cause colon cancer by inducing O^6-methyl guanine formation and mutations of the *β catenin* gene[17]. Thus, the combined treatment with AOM and DSS is used frequently to recapitulate the molecular mechanisms underlying CAC.

A transcription factor, NF-κB, is a key player in

Table 1 Pathological changes in various mice after azoxymethane/dextran sulfate sodium treatment

	Ingested DSS concentration	Body weight loss (> 20%)	Granulocyte infiltration (> 400/field)	Fibroblast accumulation (> 20% type I collagen$^+$ areas)	Tumor numbers
Wild-type mice	3.0%	+	+	+	> 20
CCR1-deficient mice	3.0%	-	-	-	< 5
	4.5%	+	+	+	> 20
CCR5-deficient mice	3.0%	-	-	-	< 5
	4.5%	+	+	-	< 7
CCL3-deficient mice	3.0%	-	-	-	< 5
	4.5%	+	+	-	< 5

Table is prepared according to Sasaki *et al*[22]. DSS: Dextran sulfate sodium.

inflammation and its activity is triggered by IκB kinase (IKK) complex, in response to a wide variety of pro-inflammatory stimuli such as infectious agents and pro-inflammatory cytokines[18]. Greten *et al*[19] demonstrated that IKKβ has crucial roles in AOM/DSS-induced CAC by two distinct pathways; prevention of apoptosis of epithelial cells and enhancement of growth factor expression by myeloid cells. Thus, these observations would indicate the crucial involvement of inflammatory cell infiltration in CAC development. Given the crucial roles of NF-κB in regulation of gene expression and biological functions of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and chemokines[18], we examined the AOM/DSS-induced colon carcinogenesis process by using mice deficient in the tumor necrosis factor receptor (*TNF-R*)*p55* gene[20]. We revealed that genetic ablation of the *TNF-Rp55* gene in myeloid cells resulted in reduction in intracolonic infiltration of inflammatory cells, particularly macrophages, and neovascularization, and subsequent tumor formation. Moreover, a TNF inhibitor reduced tumor progression even when it was administered after multiple tumors developed in colon. Similar phenotypes were observed by genetic inactivation of a macrophage-tropic chemokine receptor, the *CCR2* gene, in myeloid cells or the administration of CCR2 inhibitors after multiple colon tumors developed[21].

These observations prompted us to further examine the roles of another macrophage-tropic chemokine, CCL3, and its receptors, CCR1 and CCR5[22]. Mice deficient in *CCL3*, *CCR1*, or *CCR5* gene, did display marginal inflammatory reactions and subsequently develop few colon tumors when AOM was administered together with 3% DSS ingestion. All wild-type (WT) mice failed to survive 4.5% DSS ingestion, whereas CCL3-, CCR1-, or CCR5-deficient mice survived this high concentration of DSS solution with a prominent mucosal damage and leukocyte infiltration in colon. Interestingly, CCR1-deficient mice developed multiple colon tumors, whereas the same treatment caused only a small number of colon tumors in CCL3- or CCR5-deficient mice (Table 1)[22]. These observations would indicate that inflammatory cell infiltration is necessary but not sufficient for the development of CAC in this model.

The most prominent difference in pathological change is that CCL3- or CCR5-deficient mice exhibited reduced accumulation of CAFs in colon, which was evident in the later phase of CAC in this model, compared with WT or CCR1-deficient mice (Table 1). Several groups including ours further demonstrated that CAFs expressed abundantly growth factors such as hepatocyte growth factor (HGF)[23], epiregulin[24], and heparin-binding epidermal growth factor-like growth factor (HB-EGF)[22] to promote tumor cell proliferation in the later phase of this CAC model. Moreover, the deficiency of the *CCR5* gene results in reduced growth of the tumors arising from either subcutaneous or orthotopic intracecum injection of a syngeneic mouse colon adenocarcinoma cell line, colon 26. Furthermore, this attenuated tumor growth was associated with a reduction in type I collagen-positive fibroblast numbers but not inflammatory cell infiltration[22]. These observations would indicate the crucial involvement of CAFs in rather progression of CAC than its development.

ORIGINS OF CAFS

The lack of a specific marker to identify CAF[25] hampers the precise identification of the origin of CAF. α-SMA, a robust CAF marker, is also expressed by normal colonic fibroblasts under *in vitro* culture conditions[26] and other cell types such as pericytes and smooth muscle cells surrounding vasculature, and cardiomyocytes[27]. There are several additional candidate CAF markers including fibroblast activation protein (FAP)-α[28,29], S100A4/fibroblast specific protein-1[30,31], neuron-glial antigen-2, platelet-derived growth factor (PDGF) receptor-β, and prolyl 4-hydroxylase[32]. However, these molecules can be expressed by other cell types than CAFs and therefore, are not specific to CAFs. The lack of a definitive maker for CAFs, imply the phenotypical and functional heterogeneity of CAFs in CRC and this assumption is further strengthened by global gene expression profiles[33] (Figure 1).

The most possible cellular source of CAFs in CRC is resident fibroblasts in colon tissues. Supporting this notion, CAFs in liver metastatic foci of CRC exhibit the

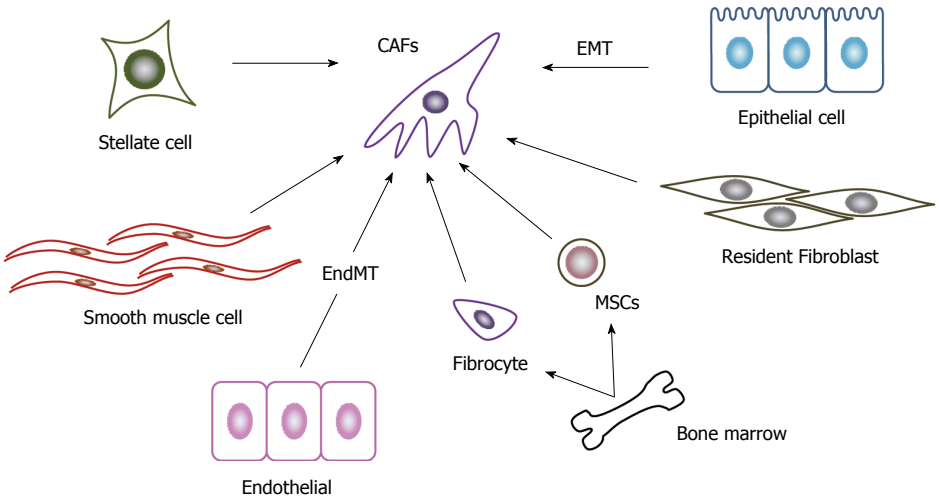

Figure 1 Origin of cancer associated fibroblasts. A variety of cells can generate to CAFs. The most important cellular source of CAFs in CRC is presumed to be resident fibroblasts. Stellate cells in intestine may be able to transform into CAFs similarly as stellate cells in liver and pancreas do. Other potential sources include epithelial cells undergoing EMT, endothelial cells undergoing EndMT, smooth muscle cells, and bone marrow-derived cells including fibrocytes and MSCs. CAFs: Cancer associated fibroblasts; CRC: Colorectal cancer; EMT: Epithelial-mesenchymal transition; EdnMT: Endothelial-mesenchymal transition; MSCs: Mesenchymal stem cells.

similar protein expression pattern as liver resident fibroblasts[34]. Kojima and colleagues demonstrated that resident human mammary fibroblasts progressively convert into CAF-like cells with enhanced α-SMA expression and pro-tumorigenic capacity, during the course of tumor progression in a breast tumor xenograft model[35]. Moreover, these cells express transforming growth factor (TGF)-β and a chemokine, stromal-derived factor (SDF)-1/CXCL12, the molecules which further initiate and maintain the differentiation of fibroblasts into CAF-like and the concurrent tumor-promoting phenotype in an autocrine and amplifying manner[35].

Stellate cells are vitamin A-containing and lipid droplet-containing cells in their quiescence state and are present in various tissues including liver, pancreas, kidney, and intestine[36]. These cells activate α-SMA expression under inflammatory and oncogenic conditions and acquire myofibroblast-like phenotypes as CAFs do. Most of hepatocellular carcinomas arise in a cirrhotic liver with prominent fibrosis. Activated hepatic stellate cells are the major source of extracellular proteins during fibrogenesis. Moreover, they can induce hepatocellular carcinoma cell growth, neovascularization, and immune evasion, to promote tumor progression[37]. Similar to hepatocellular carcinoma, pancreatic cancer is characterized by a prominent desmoplastic/stromal reaction. Like hepatic stellate cells, activated pancreatic stellate cells can produce abundantly the collagenous stroma of pancreatic cancer. Moreover, these cells can also interact closely with cancer cells to facilitate local tumor growth and distant metastasis, to mediate angiogenesis, and to induce immune evasion[38]. However, it has to be yet determined whether stellate cells in intestine, can also behave in a similar manner in the course of colon carcinogenesis, as those in liver and pancreas do.

Other types of cells are proposed to be a cellular source of CAFs, based on the analyses on other types of cancers than CRC. Accumulating evidence indicates that under chronic inflammatory conditions, epithelial cells can undergo epithelial-mesenchymal transition (EMT) to acquire myofibroblast-like phenotypes and to participate in the synthesis of the fibrotic matrix[39]. A breast carcinoma biopsy provided evidence to indicate the occurrence of EMT and a coincidental α-SMA-positive stromal reaction[40]. Moreover, when a mouse breast cancer cell line, MCF-7, was injected into nude mice, together with HBFL-1, a mammary gland epithelial cell line without tumorigenicity, HBFL-1 cells acquired myofibroblast-like phenotypes and conferred a significant 3.5- to 7-fold increase in MCF-7 tumor size in nude mice. Thus, breast cancer can transform its own nonmalignant stroma to facilitate its own growth[40]. Furthermore, when human epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (EGFR-TKI)-resistant lung cancer cells were used as a xenograft model, EMT-derived tumor cells give rise to about a quarter of CAFs, which provide cancer cells with resistance to EGFR-TKI[41]. Meanwhile, several independent groups argue against EMT as the origin of CAFs[42,43].

Bone marrow-derived mesenchymal stem cells (MSCs) are proposed to be an another origin of CAFs, because MSCs and CAFs exhibit similar immunophenotypes and share the potential to differentiate to various types of cell lineages such as adipocytes, chondrocytes, and osteoblasts[44]. Bone marrow-derived and circulating fibroblast progenitors, fibrocytes, also exhibit similar phenotypic and functional characteristics in chemically induced rat breast carcinogenesis model[45], suggesting that fibrocytes can be an origin of CAFs.

TGF-β1 can induce proliferating endothelial cells to undergo a phenotypic conversion into fibroblast-like cells with the emergence of mesenchymal markers

and the reciprocal down-regulation of CD31[46]. Moreover, when endothelial cells were irreversible tagged by crossing Tie2-Cre mice with R26Rosa-lox-Stop-lox-LacZ mice, endothelial-to-mesenchymal transition (EndMT) was evident at the invasive front of the tumors in the B16F10 melanoma model and the Rip-Tag2 spontaneous pancreatic carcinoma model[46]. Choi and colleagues further demonstrated that endothelial heat shock protein (a synonym of HSP27 in humans and HSP25 in rodents) has a crucial role in the maintenance of endothelial phenotypes and that its deficiency mediates the EndMT to accelerate fibrosis and eventually tumorigenesis in lungs[47]. Smooth muscle cells can be another source of CAFs in several cancers, particularly prostate cancer. Normal prostate stroma is enriched in smooth muscle cells, but during prostatic carcinogenesis in rats and humans, smooth muscle cells disappear with reciprocal appearance of CAFs, which can promote carcinogenesis in genetically abnormal but non-tumorigenic epithelial cells[48]. Thus, smooth muscle cells may be a source of CAFs in prostate cancer but it remains to be clarified whether CAFs originate from this population in other types of cancer, particularly CRC.

Evidence is accumulating to indicate functional heterogeneity of CAFs in various types of cancers including colon cancer[33]. This heterogeneity may arise from the differences in the origin of CAFs. Alternatively, CAFs are generated by the intricate interactions with tumor microenvironments consisting of cancer cells and other resident cells[10,25]. Thus, the heterogeneity of tumor microenvironments can induce a wide variation in CAFs. Nevertheless, this heterogeneity of CAFs can affect the clinical course of colon cancer patients[49].

RECRUITMENT AND ACTIVATION OF CAFS

Among chemokines, CCL2 and CCL3 can recruit fibroblasts[50], while CXCL12[51], CCL21[52], and CCL3[53] can recruit fibrocytes in chronic inflammation. We proved that CCL3 is produced locally at tumor sites and is associated with CAF accumulation[22,54]. Moreover, in a mouse gastric cancer model, a substantial proportion of CAFs originate from bone marrow-derived mesenchymal stem cells, which are recruited to tumor site in a TGF-β and CXCL12-dependent manner[55]. Thus, CAF accumulation may be regulated by the cooperation with chemokines and other fibroblast-tropic factors such as TGF-β.

Normal fibroblasts can inhibit *in vitro* cell proliferation of cancer cells[7]. Moreover, normal intestinal fibroblasts can maintain epithelial homeostasis to prevent carcinogenesis[23]. Thus, malignant cells must reprogram normal fibroblasts into CAFs with protumorigenic activity. This process is mediated by cancer cell-derived factors including TGF-β, CXCL12[35], PDGF[56], and interleukin (IL)-6[57]. On the contrary, several lines of evidence indicate that CAF phenotype can persist in the absence of continued exposure to cancer cell-derived factors[58]. This may be explained by the observation that CAFs increasingly acquire the capacity to express TGF-β and CXCL12, the cytokines which can act to initiate and maintain the differentiation into CAFs in auto-stimulatory and cross-communicating manner[35]. Alternatively, CAF may acquire irreversible genetic and/or epigenetic changes during the course of the differentiation, as similarly observed on tumor endothelial cells[59].

CAFS IN CARCINOGENESIS

Cancer cell growth and stemness

Human pre-malignant prostatic epithelial cells can transform to neoplastic cells when they are *in vitro* co-cultured with CAFs derived from human prostate cancer tissues[60]. Secreted factors from CAFs are believed to be responsible for this tumor-initiating capacity. Indeed, when human mammary epithelial cells are grafted into immunodeficient mice, together with fibroblasts overexpressing TGF-β and/or HGF, the engrafted cells develop into a proliferating tissue that closely resembles human ductal carcinomas[61]. These observations raise the possibility that CAFs can initiate malignant transformation of epithelial cells by secreting these growth factors (Figure 2).

Under the influence of cancer cell-derived IL-6, CAFs can secrete metalloproteinases, to stimulate EMT and cancer stem cell phenotypes in prostate cancer[62]. CAFs in colon cancer secrete HGF, activate β-catenin-dependent transcription and subsequently induce cancer stem cell clonogenicity[63]. Moreover, CAF-derived HGF also restores the cancer stem cell phenotype in more differentiated tumor cells both *in vitro* and *in vivo*. Furthermore, human colon cancer-derived and chemotherapy-treated CAFs produce abundantly interleukin-17A (IL-17A), which increases chemotherapy-resistant cancer stem cells[64].

CAFs produce various growth factors and cytokines, which can promote cancer cell proliferation; HGF[23], EGF[65], epiregulin[24], HB-EGF[22], insulin-like growth factor (IGF)[66], TGF-β[35] connective tissue growth factor[67], and CXC12[58]. Actually, most of these growth factors can be produced by cancer cells and can enhance the growth of CAFs. Moreover, CAF-derived TGF-β and CXCL12 affect CAF proliferation in an autocrine manner[35]. Thus, these growth factors form a vicious positive feedback loop between cancer cells and CAFs, and may eventually accelerate tumor progression.

Thus, CAFs can contribute to tumor development and progression by initiating malignant transformation, enhancing the proliferation of cancer cells, and inducing the cancer stem cell phenotype.

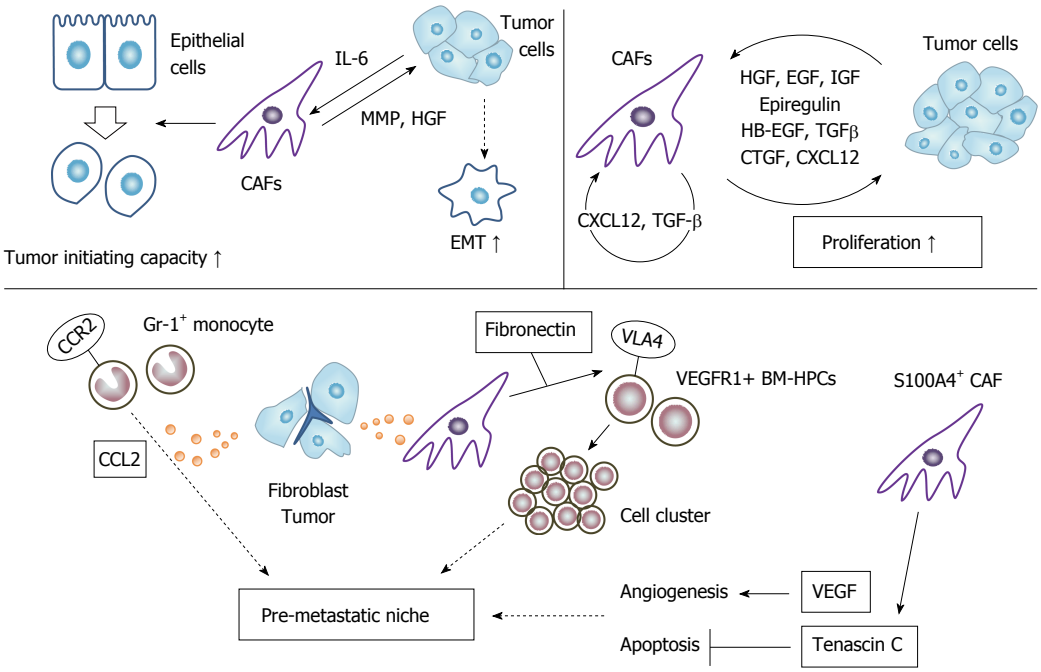

Figure 2 Action of cancer associated fibroblasts on tumor cells. CAF can induce tumor cells to enhance their tumor initiating capacity (stemness), and to undergo EMT. CAFs provide tumor cells with various growth factors to promote their growth. CAFs can also instigate pro-metastatic niche by inducing tumor cell cluster and angiogenesis, and suppressing tumor cell apoptosis. CAFs: Cancer associated fibroblasts; EMT: Epithelial-mesenchymal transition; VEGF: Vascular endothelial growth factor.

Cancer cell migration, invasion, and metastasis

Colon cancer cell lines exhibit enhanced migratory ability in a wound healing assay and increased clonogenic capacity in the presence of CAF-derived conditioned media, compared with normal fibroblast-derived conditioned media[26]. Moreover, co-injection of CAFs with a human colon cancer cell line into nude mice, significantly enhances tumor growth with increased tumor cell proliferation, compared with normal fibroblasts[26]. Gene ontology analysis further reveals that genes overexpressed in CAFs are associated with biological processes such as development (*TGFB2, PDGFC, cMET, CADM1, WNT1*) and cell-cell signaling (*TFAP2C, NTF-3, SEMA5A, EFNB2, INHBA*)[26]. These gene products can modulate the functions of cancer cells to promote their invasion and metastasis (Figure 2).

CAFs expressed various matrix metalloproteinases (MMPs) and MMP-mediated ECM degradation results in proteolytic destruction of basement membrane and aids tumor cells to invade surrounding tissues[68]. Moreover, MMP-mediated enhanced invasiveness also involves proteinase-activated receptor 1 (PAR1) expressed on cancer cell surface. CAF-derived MMP-1 cleaves PAR1 at its proper site to deliver PAR1-mediated signaling pathway in cancer cells, which is associated with cancer cell migration and invasion[69].

Fibroblasts as well as tumor cells produce abundantly CCL2, which recruits Gr-1-positive, CCR2-expressing inflammatory monocytes into lung[70]. Recruited inflammatory monocytes subsequently instigate a pre-metastatic niche, which favors lung metastasis of mouse mammary cancer. A pre-metastatic niche can be formed by direct actions by CAFs[71]. S100A4-positive CAFs produce abundantly VEGF-A, which plays an important role in the establishment of an angiogenic microenvironment at the metastatic site to facilitate colonization. Moreover, S100A4-positive CAFs produce tenascin-C to provide cancer cells with protection from apoptosis.

Gene expression-based classification systems have identified an aggressive colon cancer subtype with mesenchymal features, possibly reflecting EMT of tumor cells. Comparative analysis of stroma^{high} and stroma^{low} CRC shows that the neoplastic cells in stroma^{high} tumors express specific EMT drivers including ZEB2, TWIST1, and TWIST2[72]. Moreover, type Ⅰ collagen dominates the extracellular matrix in these aggressive colon cancer with EMT markers. Mimicking the tumor microenvironment, Matrigel enriched with type Ⅰ collagen can induce colon cancer cells to express tumor-specific mesenchymal gene, to suppress gene expression of hepatocyte nuclear factor 4, a transcriptional activator of epithelial differentiation, and its target genes, and to invade collectively patient-derived colon tumor organoids[72]. Thus, CAF-derived type Ⅰ collagen can induce EMT in cancer cells to promote their invasion.

Bone marrow-derived hematopoietic progenitor cells expressing VEGF receptor 1 (VEGFR1) home to tumor-specific pre-metastatic sites[73]. Primary tumor-derived factors induce resident fibroblast in pre-metastatic sites to express fibronectin, which interacts with VLA-4 on VEGFR1-positive cells to induce cell clusters and to promote subsequently pre-metastatic

niche formation. Fibronectin expression in fibroblasts is regulated by sphingosine-1-phosphate (SIP)-SIP receptor-STAT3 pathway[74].

CAFs can activate STAT3 pathway in cancer cells to promote malignant progression. CRCs frequently display mutational inactivation of the TGF-β pathway with elevated TGF-β production. Actually, cancer cell-derived TGF-β stimulates CAFs to secrete IL-11, which triggers gp130/STAT3 signaling in tumor cells[75]. This cross-talk can provide metastatic cells with a survival advantage.

Drug resistance

CAF-derived ECM has profound impact on cancer chemotherapy[76]. ECM forms a physical barrier and as a consequence, most anti-cancer drugs show limited penetration into solid tumors[77]. Moreover, cancer cells acquire chemoresistance through the activation of various pro-survival signal pathways including PI3K/Akt, Erk, Rho/Rock, and p53 after binding to ECM[76]. Adhesion of small cell lung cancer cells to ECM confers resistance to chemotherapeutic agents because the adhesion activates β1 integrin-stimulated tyrosine kinase to eventually suppress chemotherapy-induced apoptosis[78]. Similar mechanisms may also work in the case of resistance to radiotherapy in glioma cells[79].

In addition to ECM, CAF-derived soluble factors have been demonstrated to be involved in drug resistance. CAF-derived CXCL12 mediate drug resistance to conventional chemotherapeutics[80]. This resistance can arise from the ability of CXCL12 to promote cancer cell survival by activating focal adhesion kinase, Erk, and Akt, β-catenin and NF-κB, in CXCR4-expressing cancer cells[81].

The presence of driver mutations in receptor tyrosine kinase (RTK) pathways positions RTKs for potential targets for cancer therapy and accordingly, many anti-cancer drugs have been developed by targeting these RTKs[82]. However, RTK-mediated signals converge on common critical downstream cell-survival effectors such as PI3K and Erk, and consequently, most cells can be rescued from drug sensitivity by simply exposing them to one or more other unrelated RTK ligands. Among these RTK ligands, HGF confers resistance to the BRAF inhibitor in BRAF-mutant melanoma cells[83]. Likewise, CAF-derived HGF can confer the resistance to EGF-receptor inhibitors to human non-small cell lung cancer cells which is otherwise sensitive to the inhibitors[84].

CRC initiating cells (CICs) are resistant to conventional chemotherapy in cell-autonomous assays, but CIC chemoresistance is also increased by CAFs. Comparative analysis of matched CRC specimens from patients before and after cytotoxic treatment revealed a significant increase in CAFs after cytotoxic treatment[64]. Chemotherapy-treated human CAFs promoted CIC self-renewal and *in vivo* tumor growth associated with increased secretion of specific cytokines and chemokines, including IL-17A. Exogenous IL-17A increased CIC self-renewal and invasion, and targeting IL-17A signaling impaired CIC growth. Notably, IL-17A was overexpressed by colorectal CAFs in response to chemotherapy and this observation was validated directly in patient-derived specimens without culture[64]. These data suggest that chemotherapy induces remodeling of the tumor microenvironment through activating CAFs to secrete cytokines such as IL-17.

Tumor microenvironments

Inflammation, particularly chronic one, is closely associated with tumorigenesis of various types of cancer[12,85]. CAFs, as a major cellular component of cancer-associated inflammation, mediate tumor-enhancing inflammation by expressing a proinflammatory gene signature in an NF-κB-dependent manner[86]. NF-κB activation enhanced the expression of several chemokines such as CCL2 and proinflammatory genes such as cyclooxygenase 2 (COX-2) in CAFs. CAF-derived CCL2 mediates the recruitment of blood monocytes to tumor sites[87], favoring the generation of tumor-associated macrophages with a potent pro-tumorigenic activity. Simultaneously, COX-2 generates prostaglandin E2, which can promote both normal and malignant colonic epithelial cell proliferation[88,89] (Figure 3).

Another prominent feature of CAFs is their ability to synthesize ECM, which can serve as a reservoir for various growth factors such as TGF-β, bFGF, PDGF, HGF, and IGF-1[90]. In response to mechanical stress present in tumor tissues, CAFs exhibit an increase in contractility, which can augment the production of collagen[91]. Moreover, CAFs synthesize the specific ECM including type Ⅰ collagen, oncofetal nectin splice variants, periostin, and hyaluronan, and as a consequence, remodel ECM to promote tumor growth[90]. CAFs also express abundantly lysyl oxidase (LOX), an enzyme responsible for cross-linking type Ⅰ collagen. LOX-mediated cross-linking and the resultant tumor tissue stiffness are associated with tumorigenesis[92]. Mechanical stress activates CAFs to express members of MMPs, which regulate the degradation of ECM[90]. MMP-mediated ECM degradation can also promote cancer cell invasion[68].

Tumor microenvironment is characterized by abundant neovascularization, and this process can be induced by CAFs. CAF-induced MMP activation degrades ECM and eventually causes neovascularization[68]. Moreover, CAFs, particularly those in invasive margins, are a rich source of a CXC chemokine, SDF-1/CXCL12[58], which mediates the recruitment of endothelial progenitor cells and subsequent tumor neovascularization. Furthermore, hypoxia can induces CAFs to express a transcription factor, hypoxia-inducible factor (HIF)-1α, which in turn induces the expression of VEGF, a potent angiogenic factor[93]. VEGF production by CAFs can be further enhanced by CAF-derived IL-6, whose expression can

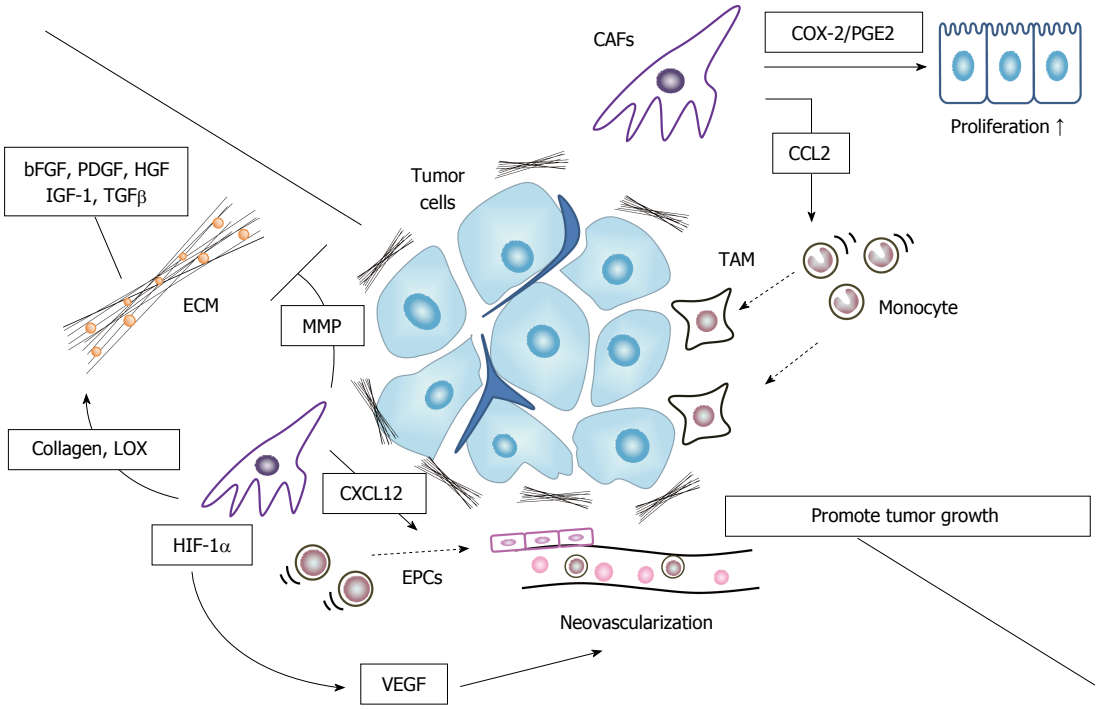

Figure 3 Cancer associated fibroblasts in tumor microenvironment formation. CAFs promote pro-tumorigenic microenvironment by producing extracellular matrix to provide tumor cells with a growth advantage, recruiting tumor-associated macrophages (TAMs) to foster tumor cell growth, and inducing neovascularization. CAFs: Cancer associated fibroblasts; EMT: Epithelial-mesenchymal transition; VEGF: Vascular endothelial growth factor; ECM: Extracellular matrix proteins; EPCs: Endothelial progenitor cells; MMP: Matrix metalloproteinase; HIF-1α: Hypoxia-inducible factor 1α.

be augmented in the presence of colon cancer cells[94].

Tumor immunity

Direct evidence on the involvement of CAFs in suppressed tumor immunity, comes from the study, which revealed that deletion of FAP-positive stromal cells enhances tumor immunity[28]. This study, however, did not clarify the cellular and molecular mechanisms in detail, but several candidate mechanisms have been proposed. CAF-derived prostaglandin E2 and indoleamine 2,3-dioxygenease can inhibit natural killer cell functions, thereby contributing to immune escape and subsequent tumor progression[95]. CAF-derived tenascin also contributes to immune suppression at tumor sites. Soluble tenascin inhibits proliferation of human T cells induced by the combination of anti-CD3 antibody and fibronectin. Tenascin further attenuates T cell proliferation driven by IL-2, while it prevents high level induction of IL-2 receptor[96]. Prostate cancer stem-like cells present in the draining lymph nodes use tenascin-C to inhibit T-cell receptor-dependent T-cell activation, proliferation, and cytokine production, and as a consequence, cancer stem-like cells are protected from T cell-mediated immune surveillance[97] (Figure 4).

CAFs abundantly express TGF-β1, which can suppress the functions of various immune cells, particularly effector T cells and natural killer cells[98]. TGF-β regulates Treg maturation and thereby suppresses immune responses. VEGF is also produced by CAFs and exhibits immunosuppressive effects[99]. VEGF can suppress the maturation of dendritic cell precursors, promote the proliferation of Tregs, and the accumulation of myeloid-derived suppressor cells (MDSC) in peripheral immune organs, and thereby inhibits T-cell immune responses.

In vivo vaccination with a DNA vaccine against FAP eliminates CAFs and eventually causes a shift of the immune microenvironment from a Th2 to Th1 polarization. This shift is characterized by increased protein expression of IL-2 and IL-7, suppressed recruitment of tumor-associated macrophages (TAMs), MDSCs, and Tregs, and decreased tumor angiogenesis and lymphangiogenesis[100]. These observations suggest the roles of CAFs in intratumor Th2 polarization and subsequent depression of tumor immunity. This Th2 polarization is mediated by CAF-derived thymic stromal lymphopoietin (TSLP), which induces *in vitro* myeloid DCs to up-regulate the TSLP receptor, secrete Th2-attracting chemokines, and acquire TSLP-dependent Th2-polarizing capability *in vitro* and *in vivo*[101]. Moreover, CD90-positive CAFs in colon cancer produce IL-6, which induces the polarization of tumor promoting inflammatory T helper 17 cells in infiltrating lymphocytes as well as the expression of cancer stem cell markers in colon cancer cells[102].

CAFs can produce a myriad of chemokines, which can attract and activate immunosuppressive cells, such as M2-polarized TAMs, MDSCs, and Tregs, thereby suppressing tumor immunity[103]. Simultaneously, CAFs can produce chemokines that promote the

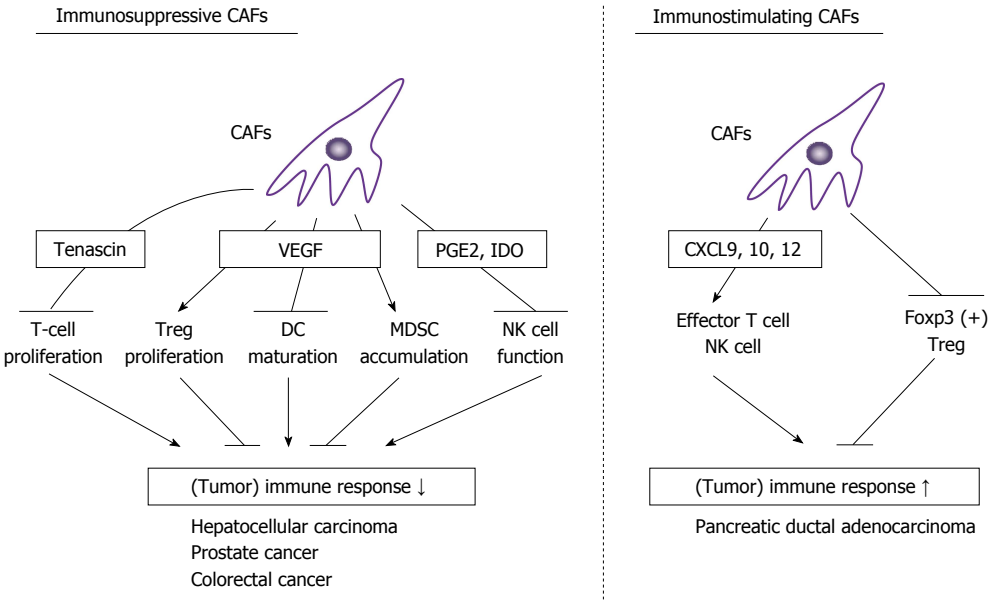

Figure 4 Double-edged actions of cancer associated fibroblasts in tumor immunity. CAFs exhibit double-edged actions in tumor immunity. In most types of cancers, CAFs can dampen tumor immunity by suppressing T cell proliferation, NK cell activity, and DC maturation, and inducing Treg proliferation and MDSC accumulation. In some types of cancers such as pancreatic ductal adenocarcinoma, CAFs can enhance tumor immunity by enhancing effector T cell and NK cell functions and depressing Treg activities. CAFs: Cancer associated fibroblasts; VEGF: Vascular endothelial growth factor; MDSC: Myeloid-derived suppressor cells; NK: Natural killer.

recruitment of effector T cells and natural killer cells, including CXCL9, CXCL10, and CXCL12[103]. If the latter chemokines are predominant chemokines produced by CAFs, they can enhance specific tumor immunity instead of suppressing it. Indeed, depletion of CAFs induces immunosuppression and accelerates pancreas cancer with reduced survival in a mouse pancreatic ductal adenocarcinoma (PDAC) model[11]. The immunosuppression is associated with increased Foxp3-positive Treg numbers and can be reversed by the immune checkpoint therapy using anti-CTLA4 antibody. Thus, in this model, CAFs restrain Tregs from expansion to keep tumor cells under immune surveillance.

CAFS AS A PROGNOSIS MARKER IN CRC

CAFs can be a useful marker to predict disease recurrence in patients with various type of cancer[10]. Similar observations are obtained on CAFs in CRC. Tumors with abundant α-SMA-positive CAFs are associated with shorter disease-free survival for stage Ⅱ and Ⅲ CRC after curative CRC surgery[104]. Likewise, high intra-tumor stroma proportion was associated with shorter overall and disease-free survival in stage Ⅱ and stage Ⅲ CRC patients after curative surgery[105]. CAFs express abundantly FAP-α and SDF-1/CXCL12. Colon cancer patients with high intra-tumor stromal FAP-α expression tend to have more aggressive disease progression and experience metastasis or recurrence[106]. Similarly, intra-tumor FAP-α and SDF-1 expression is shown to be involved in tumor re-growth and recurrence in rectal cancer patients treated with pre-operative chemo-radiation therapy[107].

The analysis was conducted on CAFs established from primary human colon cancer and revealed that CAFs exhibit significant differences in their promigratory effects on cancer cells upon co-culture with cancer cells[33]. Moreover, CAFs' promigratory effects on cancer cells are associated with fibroblast activation and stemness markers. CAF signature is identified from the gene expression signature derived from the most protumorigenic CAFs and shows a remarkable prognostic value for the clinical outcome of patients with CRC. Berdiel-Acer and colleagues conducted a transcriptomic profile of normal colonic fibroblasts, CAFs at primary tumor and CAFs at liver metastasis sites, and they identified 19-gene classifier that can predict recurrence with high accuracy in patients with CRC and correlates with fibroblast migratory potential[108]. Moreover, this 19-gene classifier can identify low-risk patients very accurately, and this identification is of particular importance for stage Ⅱ patients, especially T4N0 patients clinically classified as being at high risk, who would benefit from the omission of chemotherapy. The same group further developed a 5-gene classifier for relapse prediction in Stage Ⅱ/Ⅲ CRC by analyzing gene expression patterns in CAFs[109]. The 5-gene classifier in CAFs was significantly associated with increased relapse risk and death from CRC among stage Ⅱ/Ⅲ patients. These studies also proved the existence of heterogeneity in CAFs in terms of gene expression signatures.

Molecular classification of CRC based on global gene expression profiles have defined three subtypes;

chromosomal-instable tumor (CCS1), microsatellite-instable/CpG island methylator (CIMP)-positive tumor (CCS2), microsatellite-stable/CIMP-positive tumor (CCS3)[110]. CCS3 subtype exhibits upregulation of genes involved in matrix remodeling and EMT and has a very poor prognosis. However, a more detailed analysis revealed that their predictive power arises from genes expressed by stromal cells rather than epithelial tumor cells[111]. Moreover, functional studies indicate that CAFs can increase the frequency of tumor-initiating cells and that this enhancing effect is further augmented by TGF-β signaling. Furthermore, poor-prognosis CRC displays a gene program induced by TGF-β in tumor stromal cells. These observations would indicate that CAF-mediated gene expression profiles can be used to predict the prognosis of colon cancer patients.

CAF AS A TARGET FOR CANCER TREATMENT

Kraman and colleagues demonstrated that genetic depletion of FAP-expressing cells causes rapid hypoxic necrosis of both cancer and stromal cells in Lewis lung carcinoma-bearing mice depending on interferon-γ and TNF-α. They also demonstrated that depleting FAP-expressing cells allows immunological control of tumor[28]. However, the same group demonstrated that FAP-positive cells of skeletal muscle are the major local source of follistatin and that those in bone marrow express CXCL12 and kit ligand. As a consequence, experimental ablation of these cells causes loss of muscle mass and a reduction of B-lymphopoiesis and erythropoiesis[29]. Thus, it is probable that depletion of FAP-positive cells in tumor tissue can cause cachexia and anemia, and as a consequence, it may be difficult to target FAP to deplete CAFs.

We demonstrated the crucial involvement of the CCL3-CCR5 axis on AOM/DSS-induced colon carcinogenesis by recruiting and activating CAFs. Systemic delivery of a CCR5-antagonist-expressing vector is well tolerated by tumor-bearing mice and reduces significantly tumor mass together with decreased CAFs, when it is given even after multiple tumors develop[22]. An antagonist to another chemokine, CXCL12, inhibits CAF-mediated integrin β1 clustering at the cell surface and eventually the invasive ability of gastric cancer cells, suggesting that the inhibition of CXCL12/CXCR4 signaling in gastric cancer cells may be a promising therapeutic strategy against gastric cell invasion[112]. Moreover, CAF-derived CXCL12 can provide prostate cancer cells with the chemoresistance to a cytotoxic drug, docetaxel, and a CXCR4 antagonist can sensitize cancer cells to this drug in a subcutaneous xenograft model of prostate cancer[80]. PDAC-bearing mice frequently does not respond to immune checkpoint therapy with anti-programmed cell death ligand 1 (PD-L1) antibody despite the presence of tumor-specific CD8-positive cells, but depletion of FAP-positive CAFs uncovers the antitumor effects of anti-PD-L1 antibody and inhibits tumor growth[113]. Moreover, FAP-positive CAFs express CXCL12 and as a consequence, a CXCR4 antagonist also induces rapid T-cell accumulation among cancer cells and acts synergistically with anti-PD-L1 to greatly diminish cancer cells in pancreatic cancer model[113].

Normalization of CAFs is proposed as the strategy targeting CAFs. CAFs in prostate cancer exhibit reduced miR-15 and miR-16 expression, which is associated with the reduced post-transcriptional repression of Fgf-2 and its receptor Fgfr1[114]. The Fgf-2-Fgfr1 axis acts on both stromal and tumor cells to enhance cancer cell survival, proliferation, and migration. Moreover, reconstitution of miR-15 and miR-16 impairs considerably the tumor-supportive capability of stromal cells *in vitro* and *in vivo*[114]. Downregulation of miR-31 and miR-214 is observed in CAFs in ovarian cancer and the expression of these miRNAs induces a functional conversion of CAFs into normal fibroblasts[115]. Similar observations were also obtained on miR-31 and miR-148a expression in CAFs[116,117]. Phosphatase and tensin homolog deleted on chromosome 10 (Pten) expression in stromal fibroblasts suppresses epithelial mammary tumors. Pten-deficient mammary fibroblasts exhibit reduced miR-320 expression and reciprocally enhanced ETS2 expression, and can accelerate tumorigenicity when co-injected into mice with mouse mammary cancer cells[118]. miR-320 overexpression in fibroblasts reduces their tumorigenic activity upon co-injection with cancer cells[118]. These observations would indicate that the modulation of miRNA expression can reduce the protumorigenic capacity of CAFs, by dedifferentiating CAFs into normal fibroblasts.

Nintedanib is a broad spectrum tyrosine kinase inhibitor, with the VEGF receptor, FGF receptor, and PDGF receptor as target by binding the ATP pocket in a competitively reversible manner and is used as monotherapy for the treatment of idiopathic lung fibrosis (IPF)[119]. Nintedanib reduces lung inflammation and fibrosis in IPF as evidenced by the reduced deposition of type Ⅰ collagen and the inhibition of fibroblast activation. VEGF, FGF, and PDGF are secreted by CAFs as well as cancer cells and TAMs, and their receptors are expressed by CAFs, cancer cells, and endothelial cells[120]. Moreover, the mechanism of fibroblast activation in IPF closely resembles that in cancer[121]. Hence, nintedanib is proposed to be used as a second line therapy for non-small cell lung cancer in combination with docetaxel[122]. Another anti-fibrotic agent, pirfenidone, is used for the treatment of IPF although its exact molecular mechanisms remain enigmatic[123]. The combination of pirfenidone and cisplatin leads to increased CAF cell death and decreased tumor progression in a human non-small cell lung cancer xenografted model[124]. These observations would indicate that these anti-fibrotic

agents may be used for the treatment of cancer with abundant fibrotic changes.

Given the capacity of TGF-β as a potent fibrotic molecule[98], anti-TGF-β monoclonal antibody was developed and tested in clinical trials for several types of cancer. It can induce anti-tumor effects but simultaneously cutaneous keratoacanthomas/squamous cell carcinomas[125]. This may arise from double-edged activities of TGF-β; a tumor suppressor for normal epithelial cells and a tumor driver in tumor microenvironments[98].

FUTURE PERSPECTIVES

Accumulating evidence indicates the crucial involvement of inflammation in cancer development and progression[12]. Inflammation is a dynamic host response, wherein various types of cells participate in a concerted manner[4]. However, until recently, much attention has been focused on two processes involved in inflammation; neovascularization and inflammatory cell infiltration. Various agents have been developed as anti-cancer drugs, by targeting neovascularization, but with limited success[126]. Despite the remarkable successes of immunotherapies that modulate the adaptive immune system consisting of lymphocytes and dendritic cells[127], the plasticity and the heterogeneity of inflammatory leukocytes, monocytes/macrophages and granulocytes, have hindered the elucidation of the precise roles of these cells in carcinogenesis, and eventually the development of anti-cancer agents targeting inflammatory leukocytes[128]. Under these circumstances, fibroblasts, another type of cells in inflammation, have emerged as an important player in cancer-related inflammation[10,25,129].

CAFs express a NF-κB-dependent gene signature in mouse tumor models of skin, pancreatic and breast cancer[86]. These observations incited two independent groups to conduct fibroblast-specific deletion of the gene of *IKKβ*, which is indispensable to NF-κB activation and to examine the effects of the gene deletion on AOM/DSS-induced colon carcinogenesis[130,131]. However, the results are completely opposite to each other; Koliaraki *et al*[130] demonstrated a pro-tumorigenic activity of IKKβ whereas Pallangyo identified IKKβ as a tumor suppressor. A remarkable difference between these two groups seems to arise from the use of the different gene promoters to delete the *IKKβ* gene. Koliaraki *et al*[130] used type Ⅵ collagen gene promoter whereas Pallangyo and colleagues used type Ⅰ collagen gene promoter. The *IKKβ* gene deletion in type Ⅵ collagen-positive CAFs, decreased IL-6 production associated with decreased inflammation and suppressed tumor formation[130]. On the contrary, the *IKKβ* gene deletion in type Ⅰ collagen-positive CAFs, resulted in enhanced HGF production and subsequent tumor growth promotion[131]. Thus, CAFs as a whole may act to promote carcinogenesis but a part of them, particularly type Ⅰ collagen-positive ones, may still retain normal fibroblast-like phenotypes and functionality to suppress carcinogenesis, because normal intestinal fibroblasts can regulate intestinal homeostasis to suppress colitis-associated tumorigenesis[23].

Targeting CAFs can be a novel strategy to treat cancer, particularly inflammation-related cancer. In order to advance this strategy, however, more detailed and precise understanding of phenotypical and functional heterogeneity in CAFs is required to identify the CAF subpopulation and/or the molecules, which have crucial roles in cancer development and progression.

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TOPIC HIGHLIGHT

2016 Colorectal Cancer: Global view

MicroRNAs: Novel immunotherapeutic targets in colorectal carcinoma

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Abstract

Colorectal carcinoma (CRC) is one of the most common types of cancer worldwide and the prognosis for CRC patients with recurrence or metastasis is extremely poor. Although chemotherapy and radiation therapy can improve survival, there are still numerous efforts to be performed. Immunotherapy is frequently used, either alone or in combination with other therapies, for the treatment of CRC and is a safe and feasible way to improve CRC treatment. Furthermore, the significance of the immune system in the biology of CRC has been demonstrated by retrospective assessments of immune infiltrates in resected CRC tumors. MicroRNAs (miRNAs) are short, non-coding RNAs that can regulate multiple target genes at the post-transcriptional level and play critical roles in cell proliferation, differentiation and apoptosis. MiRNAs are required for normal immune system development and function. Nevertheless, aberrant expression of miRNAs is often observed in various tumor types and leads to immune disorders or immune evasion. The immunomodulatory function of miRNAs indicates that miRNAs may ultimately be part of the portfolio of anti-cancer targets. Herein, we will review the potential roles of miRNAs in the regulation of the immune response in CRC and then move on to discuss how to utilize different miRNA targets to treat CRC. We also provide an overview of the major limitations and challenges of using miRNAs as immunotherapeutic targets.

Key words: Colorectal carcinoma; microRNAs; Tumor microenvironment; Immunotherapy; Inflammation; Inflammatory bowel disease; Immune response

Core tip: Colorectal carcinoma (CRC) is one of the most common tumor types worldwide. Immunotherapy has been used to treat advanced CRC and has the

potential to eradicate the disease by activating immune responses. MicroRNAs play critical roles in regulating anti-tumor immune responses. There is a need to summarize the current understanding of the diverse roles of microRNAs in the regulation of immune responses and their clinical applications for CRC immunotherapy.

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INTRODUCTION

Colorectal carcinoma (CRC) is the third most common malignancy in males and the second in females, posing a serious demographic and economic burden worldwide[1,2]. Although surgical resection for patients with localized disease has dramatically improved 5-year survival rates, more than half of all patients diagnosed with CRC eventually develop recurrence and metastasis[3,4]. Current strategies such as chemotherapy are approved for the treatment of CRC; however, these non-surgical therapeutics have only modest efficacy and are ineffective against distant metastasis[5]. Moreover, traditional strategies generate reverse events, such as cytotoxicity, that limit their use[6]. Therefore, the prognosis of CRC patients remains poor and presents an opportunity to study future therapeutic approaches to improve clinical outcomes. Recently, immunotherapy has been used to treat advanced CRC and has the potential to eradicate the disease by activating immune responses[6]. Some clinical trials utilizing immunotherapy have demonstrated objective responses in CRC patients[7,8]. Moreover, immunotherapy, such as monoclonal antibody cetuximab, has immune-modulating effects in combination with traditional approaches, such as chemotherapy, thus resulting in an increase of circulating dendritic cells (DCs), natural killers (NK), central memory T cells and T-helper 1 (Th1) cells[9]. However, CRC is able to evade detection and elimination by the immune system, and the objective responses have been manifested in only a small fraction of patients with CRC[6]. For example, the lack of CRC-associated antigens limits the development of immunotherapy[6]. The important role of the immune system in the biology of CRC has been emphasized by retrospective assessments of immune infiltrates, affecting all aspects of CRC from tumorigenesis to treatment. Within the tumor microenvironment (TME), immune infiltrates can act as suppressors of tumor initiation and progression, as well as promote the proliferation, infiltration and metastasis of CRC[10]. The presence of tumor infiltrating lymphocytes, including CD8+ T cells, in the TME is strongly associated with a better prognosis[11]. Regulatory T cells (Tregs) suppress the proliferation and activation of CD4+ and CD8+ T cells and increased Tregs activation is correlated with poorer prognosis[12]. However, higher Tregs infiltration scores are correlated with overall survival, treatment-relative survival and progression-free survival. Therefore, higher Tregs infiltration score is a favorable prognostic factor in advanced colon cancer patients undergoing chemo or chemoimmunotherapy[13]. The immune evasion of CRC has complex mechanisms. The induction of immune tolerance and resistance to activated immune effectors are two important mechanisms of immune evasion[14]. Programmed death 1 (PD-1) is an inhibitory receptor that is overexpressed in nonfunctional and exhausted T cells in cancers[15]. PD-1 and the ligands for PD-1 (PD-L1) have been implicated in escape from the immune system by directly suppressing antitumor CD8+ T cells[16]. Furthermore, the recognition of tumor-associated antigens by immune effectors is one of the most important steps in antitumor immune responses. Thus, the dysregulation of antigens limits specific immune responses. For example, NY-ESO-1 is silenced in many tumor types. As a result, the immune system cannot attack cancer cells through the recognition of the antigen[17].

MicroRNAs (miRNAs) are approximately 19 to 25 nt in length and post-transcriptionally regulate gene expression in a sequence-specific manner by binding to the 3'-untranslated region, leading to either degradation of mRNA or a translation blockade[18,19]. MiRNAs have emerged as crucial modulators in various biological processes such as proliferation, tumor initiation and development[20]. Global dysexpression of miRNAs has been identified in tumors and has become a potential biomarker for tumor diagnosis, therapy and prognosis[19,21]. MiRNAs were displayed to function as oncogenes or tumor suppressors according to their target mRNAs. For example, miR-484 is significantly decreased in microsatellite instability (MSI) CRC and functions as a tumor suppressor to inhibit MSI CRC cell viability[22]. MiR-21 is upregulated in several tumors such as lung cancer, which promotes tumor cell growth and metastasis[20]. Recent studies have demonstrated that miRNAs are coming to light as a critical regulator of immune responses and its aberrant expression or dysfunction in the immune system has been associated with cancers[23-25]. MiRNAs can either inhibit or enhance immune signals by regulating the expression of the positive or negative components of immune signaling pathways. MiR-181a expression appears to control T cell sensitivity to antigens during T cell development and maturation by repressing the expression of multiple phosphatases in the TCR signaling pathway[26]. Signal transducer and activator of transcription 3 (STAT3) has been shown to play an important role in tumor-mediated mechanisms of immunosuppression[27]. MiR-124 was

found to target the STAT3 signaling pathway and exert potent therapeutic effects by enhancing immune effector responses in cancer[28]. Emerging evidence has demonstrated that immune-associated miRNAs are dysregulated in both tumor cells and immune cells, which suggests that miRNAs could be involved in communication between tumor cells and immune cells. MiR-124 is absent in all types of gliomas, and its upregulation in glioma cancer stem cells inhibits the STAT3 pathway and reverses stem cell-mediated immune suppression of T cell proliferation and Treg activation[28]. MiR-23a is upregulated in tumor-infiltrating cytotoxic T lymphocytes (CTLs) and impairs antitumor potential of patient CTLs by repressing the transcription factor B lymphocyte-induced maturation protein-1, which promotes CTL cytotoxicity and effector cell differentiation[29]. In addition to CTLs, miRNAs can regulate other immune cells, such as Th1 cells and NK cells. The members of miR-17-92 cluster miR-17 and miR-19b are the important regulators modulating Th1 responses through multiple coordinated biologic processes[30]. Moreover, miR-29 suppresses Th1 responses through the combined direct suppressive effect on T-bet, eomesodermin and interferon γ (IFN-γ)[31,32]. NK cells play critical roles in the innate immune system, contributing to the early detection and destruction of transformed cells[33]. MiRNAs have critical roles in controlling NK cell activation, survival and function[34]. Recent report has demonstrated that ovarian tumor-associated miR-20a decreases NK cell cytotoxicity by directly repressing MHC class I chain-related molecules A and B expression[35]. However, the immunomodulatory roles of miRNAs in CRC still need to be further explored. Therefore, in this review, we summarize the current understanding of the diverse roles of miRNAs involved in immune responses in CRC. We also discuss the clinical applications of miRNAs in CRC immunotherapy and the limitations and challenges in future use.

REGULATION OF IMMUNE-RELATED MIRNA BIOGENESIS IN CRC

Primary miRNAs are processed into mature miRNAs through multiple biochemical steps. Our previous review[18] and other excellent reviews[19,20,36] have illustrated the mechanism of miRNA biogenesis. Thus, we will not discuss it further here. In the process of miRNA biogenesis, the RNase III endonuclease Dicer plays a critical role in the generation of miRNAs[37]. Dicer has been reported to regulate miRNA biogenesis in Tregs[38]. Depleting miRNAs by silencing Dicer generates functional miRNAs, reduces Treg cell numbers and results in immune pathology[38]. Previous studies have found that Dicer is required, in a cell-autonomous fashion, for the development of Tregs in the thymus[38]. However, the mechanism by with the expression of Dicer in tumor cells regulates immune surveillance through miRNA processing needs to be deeply explored. Indeed, Dicer depletion upregulates intercellular cell adhesion molecule 1 (ICAM-1) and enhances the susceptibility of CRC cells to antigen-specific lysis by CTLs[39]. ICAM-1 plays an important role in physical and functional interactions between cancer cells and CTLs[40]. Furthermore, Dicer-disrupted CRC cells have downregulated expression of miR-222/339, which directly target ICAM-1 mRNA. Upregulation of ICAM-1 by repressing Dicer or miR-222/339 in CRC cells contributes to increased susceptibility of tumor cells to antigen-specific CTLs[39]. Thus, Dicer is responsible for the generation of the mature miR-222/339, which repress the expression of ICAM by binding to the 3'UTR, thereby reducing the susceptibility of tumor cells to CTL-mediated cytolysis. These findings indicate the viability of the development of novel miRNA-targeted therapy to promote cytolysis of tumor cells in tumor immunotherapy.

MIRNAS REGULATE IMMUNE RESPONSES IN THE CRC TME

MiRNAs involved in immunotherapy in CRC cells

Multiple studies have demonstrated that miRNAs play key roles in CRC tumor biology including tumor initiation, progression, invasion and metastasis, and immune responses[41-43] (Figure 1). Several miRNAs have been found to directly regulate the primary pathways of CRC initiation and development. The development of CRC follows the sequential progression from adenoma to malignant adenocarcinoma[44]. Tumor protein 53 (TP53) is frequently lost during the progression of adenoma to adenocarcinoma[45]. TP53 loss mediates apoptosis and cell cycle arrest through the inhibition of a direct downstream target, miR-34a[46]. Increased miR-17-92 cluster expression from adenoma to adenocarcinoma is associated with the transcriptional activity of c-myc, which induces the expression of miR-17-92[47]. Uncontrolled growth and proliferation are key components in the colorectal tumorigenesis. The dysfunction of the PI3K pathway and its associated proteins plays important roles in the cell cycle regulation of CRC. MiR-144 has been found to inhibit the PI3K pathway by negatively regulating mTOR[48]. Emerging evidence has demonstrated that miRNAs are most frequently associated with the invasion and metastasis of CRC. Upregulation of miR-21 positively regulates invasion and metastasis by directly targeting several tumor suppressor genes, including programmed cell death 4 (PDCD4)[49] and phosphatase and tensin homolog (PTEN)[50] in CRC. MiRNAs could regulate CRC cell invasion and metastasis through the epithelial to mesenchymal transition (EMT). Activation of EMT allows CRC cells to migrate and metastasize to distant organs. Recent studies suggested that miRNAs are new EMT regulators and regulate CRC metastasis.

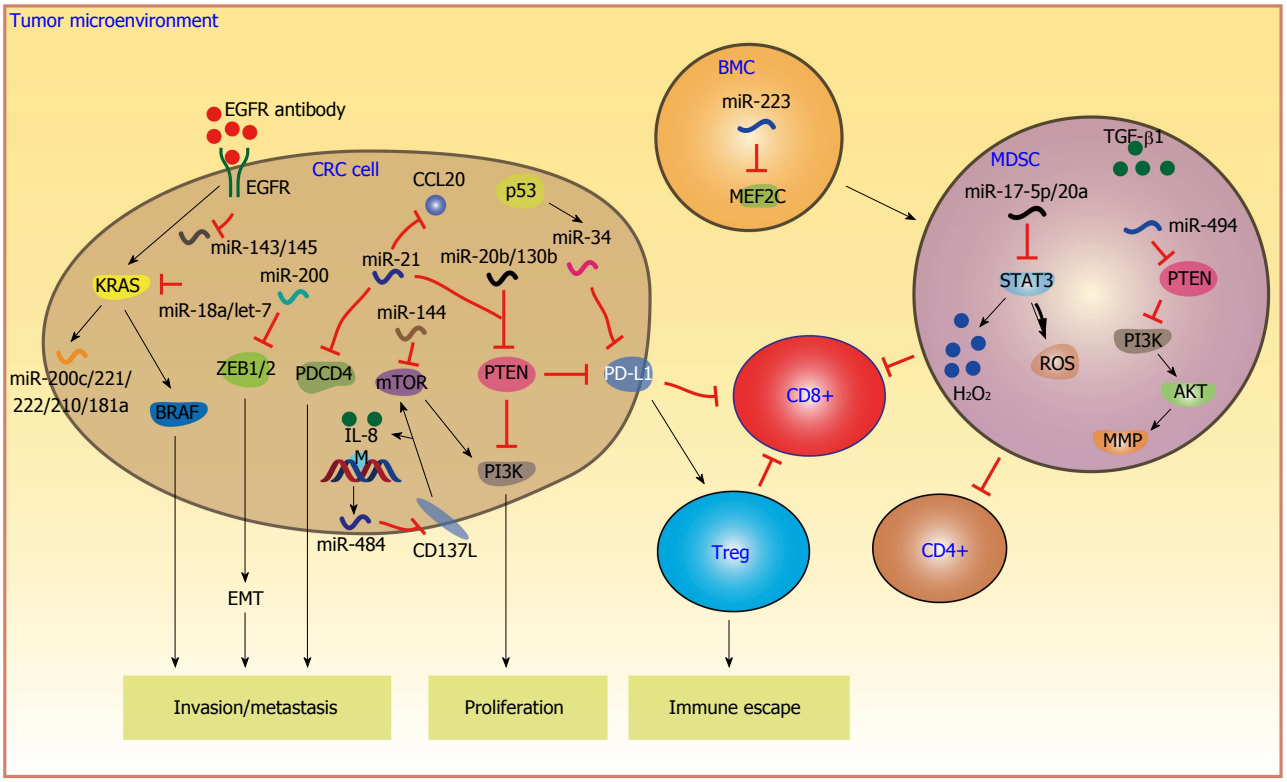

Figure 1 MicroRNAs control colorectal carcinoma progression through modulating anti-tumor immune responses in tumor microenvironment. MiRNAs play important roles in colorectal carcinoma (CRC) biology, including tumor proliferation, invasion/metastasis and immune responses. For example, miR-20b and miR-130b repress the expression of PTEN and increase the activity of PI3K, leading to the increased viability of CRC cells. MiR-21 promotes CRC invasion and metastasis by directly targeting PDCD4. Furthermore, miR-34a enhances CD8+ T cells cytotoxicity by repressing the expression of PD-L1, contributing to the elimination of CRC cells by CTLs.

Downregulation of the miR-200 family has been found to induce EMT by targeting zinc finger E-box binding homeobox 1/2 (ZEB1/2) in CRC cells, which results in the development of CRC metastasis[51-53]. Therefore, the dysregulation of miRNAs is involved in the full range of CRC development.

According to previous reports, the immune system plays complex and multi-faceted roles in CRC, affecting all aspects of CRC initiation and metastasis[10]. The body's immune system can act both to suppress tumor initiation and progression, as well as promote malignancies. During tumorigenesis, normal cells transform into neoplastic cells through multifactorial processes that involve genetic and epigenetic factors, leading to the immune escape of tumor cells. Immune escape plays a key role for the success of tumor cell growth and survival through developing multiple suppressive mechanisms in the TME[54]. More recently, miRNAs have been observed to play important regulatory roles in the immune system. The abnormal expression of co-inhibitory molecules of PD-L1 in the TME has been identified as an important immunosuppressive mechanism in many tumor types, and contributes to tumor immune escape by inhibiting T cell activation and proliferation[55]. Furthermore, PD-L1 expression in tumor cells may be associated with the expansion of Tregs in the TME, which suppresses the activation and proliferation of effector T cells[16,56,57]. MiR-20b, -21 and -130b are upregulated in advanced CRC and inhibit PTEN expression, resulting in PD-L1 overexpression[58]. PTEN, a famous tumor suppressor gene, plays a critical role in CRC cell growth, proliferation and the occurrence of CRC[59]. This study revealed a novel mechanism of PD-L1 protein expression mediated by miRNAs indirectly, which leads to the immune escape of CRC. Moreover, miR-34 has been found to modulate tumor escape by directly targeting PD-L1 in lung cancer[60]. MiR-34a therapy elicits the activation of tumor-infiltrating immune cells (CD8+ T cells) and decreases exhausted tumor-infiltrating immune cells (PD1/CD8+), indicating an active immune response and reduced tumor tolerance[60]. These results suggest that the miRNA-PD-L1 axis may be a therapeutic target for CRC immunotherapy and miRNAs may be implicated in checkpoint blockade therapy.

Cancer immunotherapy has the potential role to eliminate tumor cells by eliciting immune responses through the recognition of tumor-specific antigens on tumor cells. Numerous studies have reported that monoclonal antibody (mAb)-based immunotherapy against tumor antigens is a promising strategy and has been clinically effective as CRC therapeutics[54]. Antibodies targeting tumor antigens block major

pathways central to tumor cell proliferation and survival. Anti-human epidermal growth factor (anti-EGFR) antibodies, such as cetuximab and panitumumab, have been approved and routinely used for the treatment of CRC[54]. EGFR, commonly overexpressed in CRC, leads to the stimulation of the oncogene KRAS that subsequently activates BRAF, which contributes to the promotion and progression of a broad spectrum of CRC[61]. These two antibodies can trigger tumor antigen-specific cellular immunity to eliminate CRC cells. Recent studies have demonstrated that miRNAs could serve as predictive or prognostic biomarkers for anti-EGFR therapy in patients with wild-type KRAS (wt-KRAS) metastatic CRC (mCRC). Anti-tumor immune responses of antibody and EGFR signaling pathway have been shown to be regulated by miRNAs. MiR-31-3p/5p have been confirmed to be strongly associated with time to progression in wt-KRAS mCRC patients treated with cetuximab but not panitumumab, suggesting that miR-31-3p/5p should not be used as a predictor of response to panitumumab[62]. Two reasons could explain the predictive role of miR-31-3p/5p for cetuximab: one associated with concomitant chemotherapy, and the other with different immune responses linked to cetuximab and panitumumab[54]. Furthermore, the expression level of miR-31-3p is associated with progression-free survival, indicating that miR-31-3p seems to be a new mCRC biomarker for anti-EGFR therapy[63]. Significant miR-31* upregulation and miR-592 downregulation show a marked difference in progressive disease compared to disease control[64]. Moreover, the upregulation of some miRNAs, such as let-7 and miR-140-5p, as well as the downregulation of miR-1224-5p are significantly associated with poor overall survival, revealing that in mCRC patients with wt-KRAS/BRAF, miRNA profiling can efficiently predict the prognosis after anti-EGFR treatment[64]. Interestingly, miRNAs can also directly or indirectly target EGFR signaling pathway and its signaling components. Therefore, miRNAs-EGFR signaling has been validated as a therapeutic target in CRC. EGFR has been found to control and negatively regulate the expression of miR-143 and miR-145 in CRC cell lines, two famous tumor suppressive miRNAs, which suggests that inhibition of EGFR may upregulate the expression of these two miRNAs and suppress tumor growth and progression[65]. According to previous reports, wt-KRAS, a major factor that drives CRC progression, is a necessary condition for anti-EGFR treatment and it can be activated by the stimulation of EGFR[66]. Several miRNAs, miR-143, miR-145, let-7 and miR-18a*, act as tumor suppressors and repress the expression of KRAS[61]. In contrast to targeting KRAS, KRAS can regulate the expression of miRNAs. Recent studies have demonstrated that KRAS upregulates the expression of some oncogenic miRNAs, including miR-200c, miR-221/222, miR-210 and miR-181a[67]. These miRNAs act as oncogenic miRNAs and promote CRC progression. Therefore, the extensive involvement of miRNAs in regulating EGFR signaling pathway and its components suggests that miRNAs could serve as promising predictive biomarkers and therapeutic targets for anti-EGFR therapy in CRC.

MiRNAs regulate stromal cells

Tumor tissues have two distinct regions: the tumor bed and TME. TME comprises fibroblasts, various immune cells and endothelial cells, along with extracellular matrix, proteases and cytokines[68]. TME represents an integral part of tumors and establishes dynamic interactions with tumor cells that influence tumor growth, metastasis and immune tolerance. Stromal cells, including cancer-associated fibroblasts (CAFs), myeloid-derived suppressor cells (MDSCs) and mesenchymal stem cells (MSCs), are important components of TME and can shape anti-tumor immunity and responsiveness to immunotherapy[68]. MDSCs are a heterogeneous group of myeloid cells in early differential stages and one of the major elements of the immunosuppressive network responsible for effector T cell defective response in cancer[69]. A previous report has found that the number of blood MDSCs correlates with the stage and metastasis in many tumor types, including CRC[70-72]. MDSCs could inhibit the host immune response in tumor cells by interfering with immune cell responsiveness to interferons α and γ in CT26 bearing mice[70]. Although MDSCs contribute to immune invasion *via* suppression of anti-tumor functions of CD4+ and CD8+ T cells, the molecular mechanisms involved in the expansion, function and infiltration of MDSCs are not fully understood. Numerous studies have demonstrated that miRNAs play key regulatory roles in MDSC development, expansion and function. Recently, miR-17-5p and miR-20a have been found to alleviate the suppressive potential of MDSCs in the CT26 colon carcinoma model[73]. The overexpression of miR-17-5p and miR-20a remarkably reduces the expression of reactive oxygen species (ROS) and the production of H_2O_2 by modulating the expression of STAT3. Furthermore, MDSCs transfected with miR-17-5p and miR-20a are less able to suppress antigen-specific CD4+ and CD8+ T cells. Thus, the modulation of miR-17-5p and miR-20a expression may play important roles for the process by which patients with a tumor can overcome the immune tolerance mediated by MDSCs and could potentially be applied in immunotherapy against CRC. Increased expression of miR-17-5p and miR-20a, which are members of the miR-17-92 cluster, can promote tumor development in cancer cells. However, miR-17-92 members block the immunosuppressive function of MDSCs by silencing STAT3 expression. These studies indicate that the functional diversity of miR-17-92 cluster may result from the different targets subjected to miR-17-92

post-transcriptional silencing in different cell types, different developmental or physiological contexts[73]. MiR-494, whose expression is mainly upregulated by TGF-β1, has been identified as an essential player in the regulation of the accumulation and activity of MDSCs by targeting PTEN and activating the PI3K/AKT pathway[74]. Upregulation of miR-494 represses PTEN expression, resulting in increased activity of AKT pathway, and facilitates tumor cell invasion and metastasis by upregulating of MMPs in murine breast and colon cancers. These findings revealed that TGF-β1-induced miR-494 expression in MDSCs plays a critical role in regulating the accumulation and functions of MDSCs and may be identified as a potential target in cancer immunotherapy. Emerging studies have demonstrated that CD11b+Gr1+ MDSCs play an important role in the suppression of T cell responses and the induction of T cell tolerance in tumors[75]. MiR-223 has been identified to be related to myeloid development in myeloid expansion and differentiation[76]. The expression of miR-223 was downregulated during the differentiation of CD11b+Gr+ MDSCs from bone marrow cells (BMCs) upon exposure to tumor-associated factors. Moreover, overexpression of miR-233 remarkably inhibits differentiation of BMCs into MDSCs by targeting myocyte enhancer factor 2c, which promotes the accumulation of MDSCs.

In addition to regulating stoma cells, miRNAs also play key roles in modulating the expression of cytokines or chemokines in TME. Chemokines, which are chemo-attractant cytokines and are primarily known as critical regulators of the immune system, have been implicated in malignant transformation and tumor progression[77]. In the TME, specific chemokines have been found to be responsible for the recruitment of leukocytes into tumor sites[78]. In addition, chemokines also play an important role in the functionally of tumor cells and other cells in TME, which together modulate the processes of tumor development and progression[79]. Emerging evidence has demonstrated that various miRNAs can regulate different chemokines in the TME of CRC. Chemokine CCL20 was shown to regulate CRC progression and metastasis through binding to its receptor CCR6[80]. Moreover, CCL20 and its receptor CCR6 are significantly increased in CRC[81]. Interestingly, miR-21, a well-known oncomiR, has been identified to target CCL20 in CRC cells[82,83]. Ectopic expression of miR-21 downregulates CCL20 gene expression in different cell lines. These results seem to indicate that miR-21 acts as a tumor suppressor in this situation. However, for functional interaction of miR-21 with CCL20, both the two molecules must be present in the same cell. Furthermore, miR-21 and CCL20 are both significantly upregulated in CRC tissues. It is well-known that TME is composed of tumor cells and various types of stromal cells, such as fibroblasts and endothelial cells, as well as various immune cells. Thus, this suggests that miR-21 and CCL20 may not be expressed in the same cell in the TME of CRC. Actually, CCL20 expression found in CRC tissues is largely restricted to mesenchymal cells, such as macrophage and lymphocytes, in the TME[83]. Likewise, miR-21 expression is primarily detected in stromal cells, such as CAFs, and immune cells, such as macrophages, but not within tumor cells and lymphocytes in the TME of CRC tissues[83]. These data clearly exclude the chance of miR-21 and CCL20 being expressed in the same cell of TME. It may also be speculated that CCL20 secreted by infiltrated-immune cells affects miR-21 expression in CAFs through the CCR6 signaling pathway. In our recent report, we found that methylation-induced loss of miR-484 in MSI CRC promotes both cell viability and IL-8 (also known as CXCL8) production by targeting CD137L[22]. CD137, a member of the TNF superfamily, can induce cell viability by triggering the activation of the PI3K and mTOR signaling pathways[84]. Moreover, CD137L can signal back to tumor cells and induce the production of IL-8, which can promote tumor invasion by the recruitment of tumor-associated macrophages and neutrophils. However, CD137L expressed in CRC cells also functions as a co-stimulatory molecule for the production of IFN-γ by human T cells. Thus, these results may indicate that miR-484 can play a suppressive role in anti-tumor immune responses in MSI CRC through repressing the expression of CD137L. It seems to suggest that MSI is detrimental to immunotherapy for CRC treatment. MSI is the molecular feature of a mismatch repair deficient (dMMR) system[85]. Interestingly, the immunotherapy PD-1 blockade is more effective against dMMR tumors[86]. The reason may be that the increased number of mutation-associated neoantigens in dMMR tumors stimulated antitumor immune responses.

INFLAMMATION-RELATED MIRNAS IN CRC TUMORIGENESIS

Unique miRNA profiles

Chronic inflammatory disorders are often associated with an increased risk of CRC tumorigenesis[87,88]. CRC is preceded by clinically detectable inflammatory bowel disease (IBD), such as Crohn's disease (CD) and ulcerative colitis (UC)[88]. Because of chronic damage to the colon and rectum in IBD, these patients are at increased risk for CRC. Increasing evidence has demonstrated that miRNA dysregulation is an important pattern in IBD. Elevated miR-150 was found in UC and reduced the expression of c-myb, a transcription factor known to regulate the anti-apoptotic protein, BCL-2[89]. Several studies have found that increased cytokines, such as TNF, IL and interferon, cause apoptosis and play important roles in intestinal epithelial cells[90]. Apoptosis can interrupt intestinal mucosal integrity and barrier function and eventually result in inflammation[91]. MiR-29a has been

found to promote intestinal epithelial apoptosis in UC by directly targeting myeloid cell leukemia 1, which is an anti-apoptotic BCL-2 family member and essential for the survival of multiple cell lineages[92]. Although both CD and UC are subtypes of IBD, the expression patterns of miRNAs show region-specific differences and are expressed differently. Eight different miRNAs could be used to distinguish active CD from active UC[93]. Moreover, the expression of miR-23b/106/191 was increased in active UC tissues, while miR-19b/629 were decreased in Crohn's colitis patients[94]. Given these common changes in miRNA expression profiles in IBD, these miRNAs may be involved in regulating central components of the immune system.

MiRNAs regulate inflammatory signaling pathways

Numerous studies have demonstrated that miRNAs can regulate many inflammation associated pathways in IBD. The IL-6-STAT3 signaling pathway plays an important role in IBD and specifically STAT3 is up-regulated in UC[95,96]. This pathway regulates the development of UC and has been implicated in the progression of UC to CRC[97]. Recently, miRNAs have been identified as a potent regulator of STAT3 activity in human colonocytes. MiR-124 is downregulated in children with active UC, resulting in increased levels of STAT3 expression and the transcriptional activation of its downstream targets, which could promote inflammation and the pathogenesis of UC in children[98]. The vitamin D receptor (VDR) is highly expressed in the epithelial cells of the gut mucosal epithelial barrier and plays a critical role in maintaining the integrity of the mucosal epithelial barrier by suppressing inflammation-induced intestinal epithelial cell apoptosis[99]. Epithelial VDR levels are reduced by more than 50% in IBD and promote colitis[99]. MiRNAs have been identified to be involved in the downregulation of VDR. Pro-inflammatory cytokine TNF-α suppresses epithelial VDR expression through induction of miR-346[100]. TNF-α is one of the most important cytokines in regulating the development of colitis, and anti-TNF therapy is a standard therapy in the management of IBD[101,102]. Thus, the use of TNF-α, which as an inflammatory mediator inhibiting VDR, will have promising clinical applications. Further, TNF-α is markedly increased and may serve as an objective index of inflammatory activity in human UC. A recent report has found that miR-19a, which is downregulated in UC, directly targets the expression of TNF-α, and induces the expression of downstream inflammation targets IL-8 and granulocyte-macrophage colony-stimulating factor[103]. Immune dysregulation is thought to play a critical role in the pathogenesis of CD[104]. Cytokines are involved in immune regulation and the dysregulation of cytokines contributes to heightened inflammation[105]. Therefore, cytokine signaling pathways may be useful targets for CD treatment. MiR-19b has been reported to regulate cytokine signaling by directly targeting suppressors of cytokine signaling[106]. SOCS proteins are important physiological regulators of immune responses and negatively regulate cytokine expression through the JAK/STAT pathway[107]. Thus, this study will provide potential evidence for the development of miR-19b-based anti-inflammation therapies for CD treatment. Neurotensin (NT) is a neuropeptide expressed in the central nervous system and the intestine, while NT receptor 1 (NTR1), the high-affinity receptor of NT, is expressed in neurons and colonic epithelial cells[108]. In the colon, the NT/NTR1 signaling pathway can promote inflammation in acute colitis and their expression increases in patients with UC[109]. NT induces miR-133a expression in colonic epithelial cells[110]. Moreover, miR-133a modulates NT-induced MAP kinase and NF-κB activation, as well as transcription of pro-inflammatory cytokines, such as IL-6/8/1β, by inhibiting aftiphilin (AFTPH) expression[110]. This study may represent a promising strategy to inhibit miR-133a and restore AFTPH expression for the treatment of IBD.

MiRNAs regulate immune responses

Immune responses play important roles in connecting inflammation and CRC. IBD has been connected to predisposing genetic factors that contribute to abnormal immune responses. For example, adaptive immune system related genes that have been linked to IBD include components of the IL-23 signaling pathway, which are required for induction of Th17 cells[111,112]. Interestingly, a recent study has found that the intracellular sensor nucleotide-binding oligomerization domain protein 2 (NOD2) can induce miR-29 expression in human DCs to limit IL-23 release[113]. NOD2 is required for the induction of miR-29; miR-29 downregulates IL-23 and attenuates Th17 cell responses in CD. Activation of NOD2 has a key role in influencing the release of pro-/anti-inflammatory cytokines, particularly IL-1belta/6/8/23[114,115]. Furthermore, activation of NOD2-dependent pathways contributes to the induction of NF-κB, as well as the activation of JNK and p38 MAPK[116,117]. NOD2 not only induces miRNA expression but also has been identified as a functional downstream target of several miRNAs. MiR-122[118] and miR-192[119] can directly target NOD2 and suppress the expression of NOD2. Ectopic expression of these two miRNAs inhibits NOD2 expression, NF-κB activation and immune responses. Furthermore, miRNAs are also pivotal regulators of Th17 cells differentiation and function. MiR-210 has a particularly important role in regulating Th17 cell lineage, and is a signature miRNA of hypoxia[120]. The expression of miR-210 increases rapidly in hypoxia-challenged Th17 cells[120]. Hypoxic conditions inhibit degradation of the subunits of hypoxia-inducible factor 1 (HIF-1) and lead to their stabilization[121,122]. HIF-1 plays important roles in regulating Th17 differentiation[123].

Intriguingly, miR-210 directly targets HIF-1α, reduces HIF-1α transcript abundance and the proportion of cells that produce inflammatory cytokines, and controls IBD severity[120]. In addition to regulating Th cell differentiation, miRNAs can regulate leukocyte migration by targeting chemokines. MiR-141 regulates colonic leukocyte trafficking *via* directly targeting CXCL12β during intestinal inflammation of CD[124]. The miR-141-CXCL12β pathway contributes significantly to the development of CD, likely by affecting the recruitment of immune cells into the inflamed colonic tissue. Therefore, repressing colonic CXCL12β and immune cell recruitment with miRNAs represents a feasible and promising strategy that may be valuable for the treatment of CD. Moreover, the expression of a number of intestinal epithelial cell-derived chemokine is regulated by miRNAs. MiR-192 was dramatically upregulated in UC and regulated the expression of macrophage inflammatory peptide-2α (MIP-2α), which is a chemotactic chemokine secreted by colonic epithelial cells and macrophages[125]. Recently, miRNAs have been found to regulate gut mucosal immunity *via* epithelium-T-cell crosstalk. MiR-375 regulates gut mucosal immunity by modulating the differentiation of goblet cells, and targets the expression of thymic stromal lymphopoietin and resistin-like molecule-β, a Th-2-facilitating epithelial cytokine and an important goblet cell effector, respectively[126].

IBD and CRC are related to dysregulation of the intestinal immune system and of the microbiota[127]. The gut microbiota provides constant immunological signals to intestinal tissues, thus a variety of regulatory mechanisms have evolved to ensure proper development and function of the intestinal immune system. MiRNAs have been demonstrated to regulate expression of genes involved in microbial recognition and downstream immune activity. Thus, miRNAs play a key role within the intestinal immune system during its interactions with the gut microbiota[128]. Nine miRNAs are differentially expressed in the colon of germ-free mice colonized with the microbiota; the upregulation of miR-665 significantly downregulates the ATP-binding cassette sub-family C member 3[129], which belongs to the multidrug resistance-associated protein family[130]. MiR-146a represses a subset of gut barrier and inflammatory genes and restricts the expansion of intestinal T cell populations, including Tregs, Th17 and T follicular helper cells[131]. Furthermore, the regulatory effect of microbiota on miRNA expression and on the maintenance of intestinal homeostasis has been investigated. The expression of miR-10a and its targets IL-2/IL-23p40 play important roles in regulating innate immune responses to commensal bacteria in DC[132]. These studies open new perspectives for the investigation of miRNA regulation in intestinal diseases and microbiota. Furthermore, a variety of bacterial infection can trigger inflammasome formation and activation[133]. Inflammasomes are large cytosolic protein complexes that promote immediate inflammatory responses and regulate intestinal homeostasis through its effects on the intestinal microbiota[134,135]. The inflammasomes have potentially critical roles in the development and pathogenesis of IBD, because of their important role in intestinal immunity[135]. Emerging evidence has demonstrated that inflammasomes can influence intestinal microbiota. Some reports have exhibited that mice lacking various components of the inflammasome showed prototypical alterations in their microbiota, predisposing these mice to the development of IBD[136]. Inflammasomes have important roles in IBD, whereas the mechanisms of inflammasome formation and activation in the onset of intestinal diseases have not yet been fully established. MiRNAs may be regulators in inflammasomes formation and activation. Although only few reports explore the role of miRNAs in modulating inflammasomes in intestinal diseases, some works have been carried out in other disease types[137,138]. In the future, more investigations should illustrate novel understandings on miRNA and inflammasome interactions, which will potentially lead to the discovery of new treatment approaches for intestinal disease or CRC.

PERSPECTIVES AND CHALLENGES

The limitations of surgery and chemo/radio therapies to treat CRC patients necessitate the development of novel strategies, including immunotherapy. Tremendous progress has been made in understanding the role of the immune system in driving the development of CRC. Immune cell markers could be used to predict CRC outcomes[11], while targeting various aspects of the immune system could be used to generate anti-tumor immune responses. Unfortunately, effective immunotherapy in CRC treatment remains elusive. The ultimate goal of immunotherapy is to recruit the immune effectors to destroy cancer cells. However, immune escape leads to the failure of immunotherapy. It has been proposed that cancer cells use immune escape to evade the recognition and killing by the host immune system. For example, the expression of B7, a co-stimulatory molecule, has been found to be involved in CRC progression and immune escape mechanism[139]. Furthermore, STAT3 regulates key pathways that mediate immune escape in the TME[140,141]. Therefore, novel approaches need to be identified to overcome immune escape. Although several trails utilizing cancer vaccines or immune-checkpoint therapies have demonstrated objective responses in immunized patients with mCRC, more work is needed.

MiRNAs are deregulated and play critical roles in CRC. Initially, miRNAs have been found to act as oncogenic or tumor suppressor genes and regulate tumorigenesis, angiogenesis, progression, invasion and metastasis. The discoveries of miRNAs have

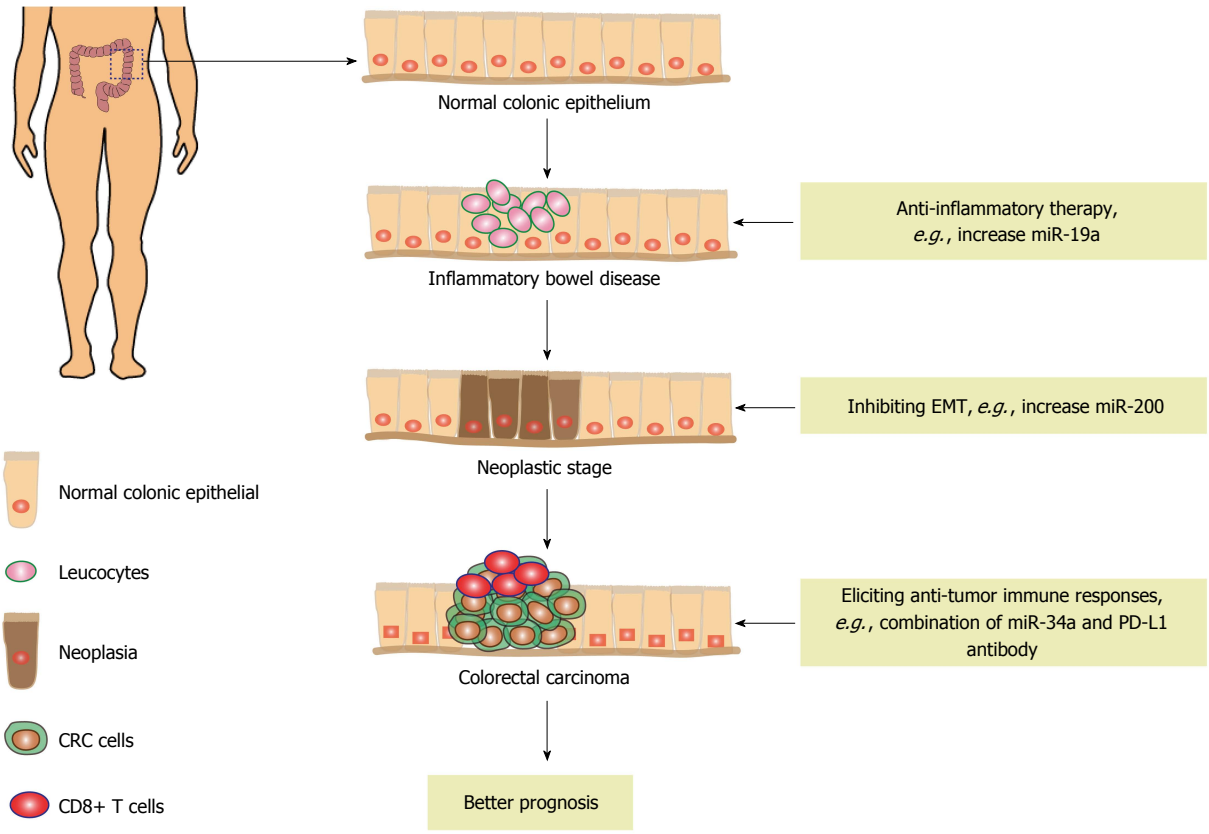

Figure 2 MiRNA-based immunotherapy in multi-steps colorectal carcinoma tumorigenesis. Various immune-related miRNAs have been identified that are dysregulated during colorectal carcinoma (CRC) tumorigenesis. These findings can be used to prevent and treat CRC in different pathological stages. In IBD stage, miRNAs and their specific targets are ideally anti-inflammatory targets. For example, overexpression of miR-19a inhibits inflammatory response through targeting TNF-α. During the stage that normal colonic epithelium transforms to neoplasia, miRNA can be utilized to suppress EMT. For example, increased miR-200 reverses EMT and represses the neoplastic cells. In the CRC stage, in addition to traditional approaches, combination of immune-related miRNAs and existing immunotherapeutic strategies can elicit anti-tumor immune responses. For example, combination of miR-34a and PD-L1 antibody can eliminate tumor cells through eliciting tumor-infiltrating CD8+ T cells. The patients with CRC receiving miRNA-based immunotherapy will have better prognosis.

opened a new era of CRC research and the potential of novel therapies for CRC treatment (Figure 2). Recently, miRNAs have been identified to regulate immune responses in CRC. The fact that miRNAs regulate tumor behaviors through targeting multiple genes, including immune associated genes, makes them excellent candidates for gene therapy. Therefore, the ability to use miRNA-based therapy to restore a dysfunctional immune system to a healthy state needs to be deeply studied. Generally, restoring the expression of tumor suppressor miRNAs or inhibiting the expression of oncogenic miRNAs has been employed for the development of miRNA-based therapeutics. In this regard, an example such as inducing miR-34a expression has been found to elicit tumor-infiltrating CD8+ T cells activation by targeting PD-L1[60]. Actually, an miR-34 mimic (MRX34) has become the first miRNA to enter phase I clinical trial (NCT01829971). The study was started to evaluate the safety of MRX34 in patients with unresectable primary liver cancer or other selected solid tumors, such as renal cell carcinoma, non-small cell lung cancer and melanoma. Targeted therapy and immunotherapy, such as PD-1 antibodies, in melanoma have led to a marked improvement in patients' survival and their quality of life[142]. Therefore, the miRNA-target mRNA axis may be another novel immunotherapeutic target for cancer treatment. For example, STAT3 has been identified to play a pivotal role in a wide variety of tumor-mediated immunosuppression and is one of the first candidates that researchers assessed for potential miRNA binding. MiR-124 can enhance immune effector responses by directly inhibiting the activation of the STAT3 signaling pathway[28]. Because aberrant activation of STAT3 pathway occurs in each stage of CRC tumorigenesis, including inflammation, adenoma and carcinogenesis, targeting diverse miRNA-STAT3 axes in specific pathological stages may improve therapeutic efficacy. In addition, combinational therapies are promising approaches for CRC immunotherapy. As shown above, PD-L1 is the target of miR-34. MiR-34 therapy and PD-L1 monoclonal antibody therapy combinations will elicit stronger anti-tumor immune responses. Furthermore, miRNAs are known to target networks of genes and this may provide a unique therapeutic advantage. Therefore, altering the expression of multiple miRNAs simultaneously by repressing distinct targets is a potential approach for CRC treatment.

MiRNAs exhibit diverse immunomodulatory effects through their action on multiple mRNA species because they control many important immunological processes. Although great efforts have been made to discover the precise role of miRNAs in anti-tumor immune response regulations, there are many challenges for the development of miRNA-based immunotherapy. These challenges include screening tissue-specific profiles, target identification, safe delivery to specific tissue, off-targets and biological safety. According to previous reports, miRNA expression is deregulated in tumor tissues compared to normal tissues and each patient has specific miRNA profiles. Thus, the ability to identify distinct miRNAs to treat CRC needs to be deeply explored. Moreover, although many computational algorithms have been developed to identify potential targets, many critical functional miRNA targets could not be identified due to the inability of bio-informatics predictions to find miRNA binding sites with seed mismatches[143]. Fortunately, novel approaches have been developed to overcome these limitations. The major challenge in miRNA-based immunotherapy is the safe delivery to specific tissues without side effects. Moreover, there are challenges to ensure miRNAs to reach the TME rather than other organs. To overcome these delivery and biological safety hurdles, some effective and marketable strategies, such as viral and non-viral strategies, have been developed[144,145]. However, novel approaches are needed to determine the efficacy of immune responses in clinical applications. In addition, another obstacle faced is whether exogenous miRNAs can precisely regulate their appeal targets in tumor cells. Therefore, there is a long way to go before we resolve many technical and biological challenges. Nevertheless, the successful development of miRNA-based immunotherapy has attracted the attention of many researchers.

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TOPIC HIGHLIGHT

2016 Colorectal Cancer: Global view

Treatment dilemmas of cetuximab combined with chemotherapy for metastatic colorectal cancer

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Abstract

Although monoclonal antibodies (mAbs) against epidermal growth factor receptor (EGFR) have largely enriched the available therapeutic choices for colorectal cancer (CRC), the understanding and management of their associated clinical toxicities are limited. In addition, the combined strategies of administering EGFR mAbs and traditional cytotoxic agents, such as 5-fluorouracil, oxaliplatin and irinotecan, have resulted in a more complicated management of CRC treatment-related side effects compared with EGFR mAb monotherapy. We believe that a thorough recognition of the toxicities of EGFR mAb drugs is essential for physicians to increase the therapeutic index in the treatment of CRC. This review aims to summarize the existing information regarding the treatment dilemmas of cetuximab combined with chemotherapy in the management of metastatic CRC.

Key words: Cetuximab; Colorectal cancer; Metastatic; Toxicity; Chemotherapy

Core tip: The advent of epidermal growth factor receptor monoclonal antibodies (EGFR mAbs), especially cetuximab, has provided a meaningful transformation in the available treatment options for advanced colorectal cancer (CRC). Nevertheless, their efficacy is accompanied by some undesired complications. Additionally, combination treatments comprising EGFR mAbs and traditional cytotoxic agents have resulted in a more complex management of CRC treatment-related side effects. Therefore, it is imperative to understand and appropriately address the treatment dilemmas of cetuximab combined with chemotherapy for the management of metastatic CRC.

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INTRODUCTION

Colorectal cancer (CRC), one of the most commonly diagnosed malignancies, is the third leading cause of cancer-related deaths, with more than 600000 worldwide[1]. It is predicted that nearly 123 million new cases are diagnosed yearly, although the incidence has declined in long-term records[1]. Nevertheless, approximately 50% of patients have metastatic disease when first diagnosed, and among the 60% of patients with an initial curative intent, approximately 25%-40% will suffer from disease recurrence or progression[2,3]. Hence, the treatment of metastatic disease plays a key role in the management of advanced CRC, the cost of which accounts for an appreciable global healthcare burden.

Notably, with the advent of new biologic drugs during the last decade, an unprecedented surge of new treatment strategies for the management of advanced CRC has been witnessed. Consequently, the overall survival of patients with advanced CRC has been extended and their quality of life has improved significantly. According to reviews of institutional databases since 2004, which is when novel therapeutic drugs became available, the median overall survival of patients with advanced CRC has recently increased from 18 mo (95%CI: 15.8-20.2 mo) to almost 29.2 mo (95%CI: 24.3-34.2 mo)[4,5]. Moreover, the 5-year relative survival rates have changed significantly from 51% of patients diagnosed during 1975-1977 to 65% of patients treated from 2004 to 2010[1]. Therefore, considerable pharmacological advancements in recent years have transformed CRC from a disease that is rapidly lethal to one that can be managed chronically for 2-3 years (Figure 1).

Most of these new biologics should work effectively in combination with at least one of the chemotherapy regimens. The combination of 5-fluorouracil (5-Fu) plus leucovorin (LV) with the addition of irinotecan (iri) or oxaliplatin (oxa) is recommended as the primary backbone chemotherapy for advanced CRC[6,7]. Despite extensive chemotherapy treatments, even including the new biological agents, the clinical outcomes in CRC remain limited, and increasing severity of toxicity is often observed. Therefore, there is an urgent need to improve the accurate selection of treatment strategies for individuals based on the expected clinical outcomes and accompanied toxicities. Ultimately, advanced knowledge of molecular medicine might guide clinicians to select the right treatment regimen for individual patients[8]. This review aims to summarize the existing information regarding the treatment dilemmas of cetuximab (Cmab), one of the epidermal growth factor receptor (EGFR) monoclonal antibodie (mAb), combined with chemotherapy, including 5-Fu, oxa and iri, for the management of metastatic CRC.

MECHANISM OF CETUXIMAB EFFICACY

Targeting EGFR and its ligands' pathways is a promising treatment strategy because it is reported that approximately 25%-77% of CRC cases exhibited overexpression of EGFR as well as its ligands, including EGF and transforming growth factor α (TGF-α)[9,10]. Notably, mAbs targeting EGFR have had a profound beneficial effect in the treatment of CRC since the clinical application of Cmab was approved by the United States Food and Drug Administration in 2004 followed by the authorization of panitumumab (Pmab) two years later[11]. Significant improvement was achieved in the CALGB80405 trial, which showed that the OS of CRC patients reached 29.93 mo with Cmab treatment combined with chemotherapy; in particular, the OS was 30.1 mo for the Cmab and mFOLFOX6 (oxaliplatin/5-FU/leucovorin) combination[12].

Cetuximab is a human/mouse recombinant immunoglobulin G1 mAb that has a higher affinity for the extracellular domain of EGFR than other ligands, such as EGF and TGF-α. The binding of Cmab to EGFR prevents intracellular ligand-mediated receptor-related tyrosine kinase phosphorylation, resulting in the inhibition of downstream signaling pathways, including the RAS-RAF-MAPK and PI3K-Akt/mTOR pathways[13]. Consequently, the antitumor effects of Cmab are due to multiple mechanisms: (1) cell proliferation suppression: Cmab arrests the cell cycle in G1 phase and, consequently, the number of S-phase cells is decreased. This effect is a result of the increased expression of p27KIP1, a CDK2 inhibitor, and the over phosphorylation of Rb protein[14]. Then, apoptosis-associated proteins are activated, including the induction of BAX, the release of Smac and the activation of caspase 8. As a result, the number of cells arrested in G1 declines; (2) antiangiogenesis: the production of angiogenic factors is reduced by the inhibition of EGFR pathways; for example, the production of vascular endothelial growth factor (VEGF), basic fibroblast growth factor, and interleukin-8 (IL-8) is decreased, which contributes to a decline in microvessel density and enhanced endothelial cell apoptosis[15]; (3) antibody-dependent cellular cytotoxicity might be induced[16]; and (4) cancer metastasis-related matrix metalloproteinases are decreased by the inactivated EGFR. Hence, cell adhesion is reduced and metastasis is further down-regulated. The anti-tumor effect has been demonstrated in EGFR-expressing CRC cells and nude mice *in vivo*. Additionally, the combination of Cmab and chemotherapy drugs or radiotherapy exhibits significant tumor inhibition in nude mice bearing CRC cell xenografts[17].

MECHANISM OF CETUXIMAB TOXICITY

Notably, Cmab has achieved demonstrable clinical anti-cancer efficacy, and awareness of the underlying mechanism is essential in the management of advanced CRC. Cmab is a chimeric mAb against EGFR, namely, the immunoglobulin's constant region

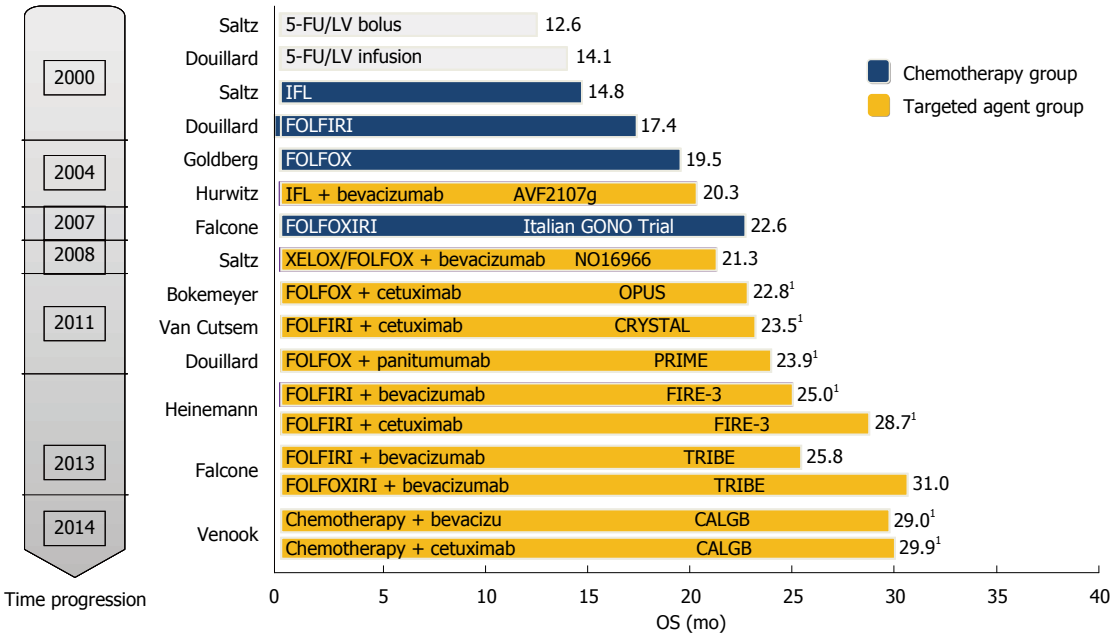

Figure 1 Development of cytotoxic and targeted agents for the treatment of colorectal cancer. [1]KRAS wide-type patients. CRC: Colorectal cancer; 5-FU: 5-fluorouracil; LV: Leucovorin; IFL: Irinotecan + 5-fluorouracil + leucovorin; FOLFOXIRI: Leucovorin + 5-fluorouracil + oxaliplatin + irinotecan.

is derived from humans, but the variable domain is derived from mice[18]. Hence, the nonhuman nature of these early antibodies leads to inflammatory reactions with repeated administrations, which might be related to the increasing immunogenicity of recipients[19,20].

According to Lee *et al*[21], to better understand of the toxicity-associated mechanism, adverse events should be distinguished into two categories: target-related (on target) and agent-related (off-target) toxicities. Generally, on-target adverse events cannot be avoided because of the specific target the agent inhibited, and they should be managed proactively. By contrast, off-target toxicities are the result of the cross-inhibition of unintended targets or cross-interaction with undesired pathways, and they are related to the specificity of the targeted agents. In addition, the pharmacokinetics are closely related to the toxicities and are determined by the inter-individual variations of drug absorption, distribution and metabolism. Consequently, ABC drug transporter polymorphisms and the cytochrome P450 genotype of the patient could be pharmacogenetic contributors to adverse events[22,23]. Importantly, it has been suggested that the mAbs could induce immune activity indirectly in a known non-allergic, cytokine-associated process of infusion reaction[24]. Chung *et al*[25] found that a high frequency of infusion reactions was significantly related to elevated circulating anti-cetuximab IgE levels pretreatment.

RECIPROCAL INTERACTIONS BETWEEN CETUXIMAB AND CYTOTOXIC CHEMOTHERAPEUTIC REGIMENS

With the increasing emergence of targeted agents, optimization of therapeutic drugs has received widespread attention, especially the choice of chemotherapy backbone and mAbs or antiangiogenic regimens. Until now, the combination of 5-Fu or oral capecitabine (cap) with either oxa (FOLFOX), XELOX, iri (FOLFIRI) or XELIRI (cap and iri) has been recommended as the standard treatment combined with targeted agents for patients with advanced CRC[7]. Hence, there is an urgent need to determine how best to integrate these regimens to achieve clinical outcomes with a high efficacy but low toxicity. To better understand the reciprocal interactions between chemotherapy regimens and targeted agents, the current study reviewed clinical trials from January 2002 and March 2015 collected from the PubMed, American Society of Clinical Oncology annual meeting, gastrointestinal cancer symposium and European Society for Medical Oncology databases; the clinical trials were phase 3 or multicenter, randomized phase 2 trials studying the FDA recommended target drugs combined with cytotoxicity chemotherapies for the first-line treatment of CRC. Research studies involving adjuvant, neo-adjuvant and maintenance regimens of CRC were excluded. Moreover, incomplete studies without safety results were also excluded.

It must be noted that Cmab has contributed greatly to improving the clinical outcome of CRC, with a prolonged OS of more than 30 mo (Figures 2 and 3, and Table 1). Of the multi-chemotherapies, FOLFIRI and FOLFOX are the most commonly used. Nevertheless, some unpredictable toxicity related to the combination occurred (Figures 2 and 3). An overview of the figures showing the adverse events of Cmab and chemotherapies reveals that the incidence of grade 3/4 hand-foot syndrome was 13%-35%,

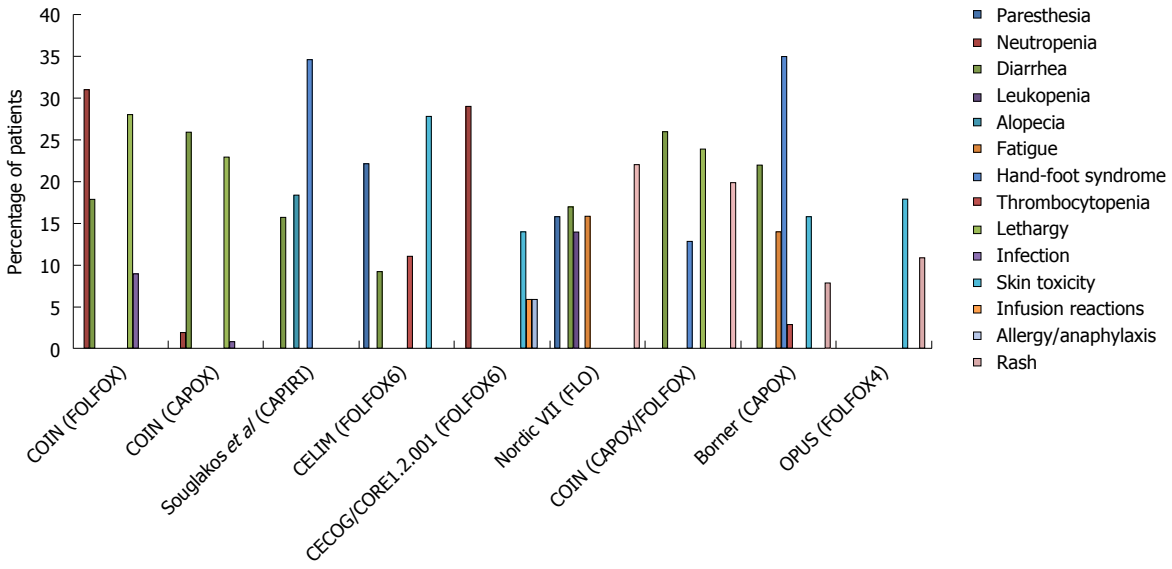

Figure 2 Toxicity of cetuximab combined with oxaliplatin-based chemotherapy summarized from the published clinical trials. FOLFOX: Leucovorin + 5-fluorouracil + oxaliplatin; CAPOX: Capecitabine + oxaliplatin; CAPIRI: Capecitabine + irinotecan; FLOX: 5-fluorouracil/leucovorin bolus + oxaliplatin.

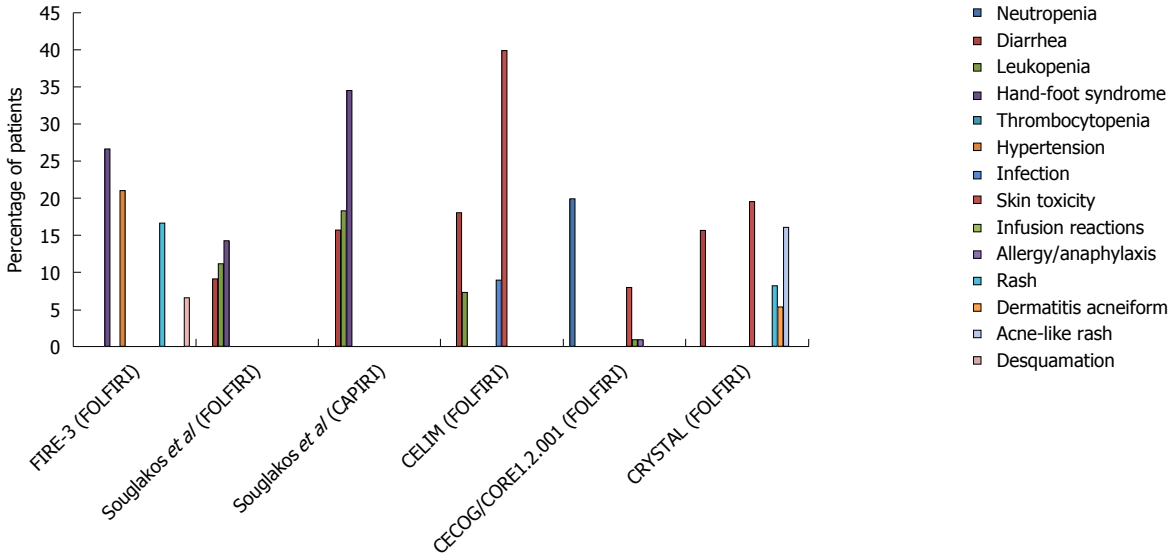

Figure 3 Toxicity of cetuximab combined with irinotecan-based chemotherapy summarized from the published clinical trials. FOLFIRI: Leucovorin + 5-fluorouracil + irinotecan; CAPIRI: Capecitabine + irinotecan.

followed by skin toxicity (8%-40%); diarrhea, neutropenia and lethargy were also common.

The efficacy and toxicity mechanism of Cmab and oxa-based chemotherapies is perplexing. In 2001, oxa was recommended as a cytotoxicity backbone for the treatment of adjuvant and advanced chemotherapy settings by the United States Food and Drug Administration[26,27]. As a conventional partner of Cmab, the regular regimens were FOLFOX in the PRIME, OPUS, and COIN trials; FLOX in the NORDIC VII trial; and XELOX in the COIN trial. However, the outcomes were distinctly different. The outcomes become more confusing when considering the results of CALGB80405, which added another layer to the already puzzling situation.

The interaction of Cmab and oxa is a double-edged sword - the interaction has shown both synergistic and antagonistic effects *in vitro*. First, the combined administration enhanced cell cycle arrest and induced cell apoptosis by elevating pro-apoptotic proteins, such as Bax and Caspase 8; meanwhile, it reduced the expression of anti-apoptotic proteins, for example, Bcl-2, and NF-κB was also decreased[28]. Second, the level of AKT phosphorylation, a target of the EGFR downstream pathway, was increased after the administration of oxa, which was apparently inhibited by Cmab[28]. Third, Cmab promoted the oxa anti-tumor efficacy by suppressing the DNA repair system, which involves increasing platinum-DNA adducts; inducing apurinic or apyrimidinic sites; and

Table 1 Cetuximab combined with cytotoxic chemotherapeutic regimens for the first-line treatment of advanced colorectal cancer

Name of study	Clinical trial phase	*n*	Chemotherapy	OS (mo)	PFS (mo)
FIRE-3[55-57]	3	171	Cmab + FOLFIRI	33.1	10.2
CALGB80405[12]	3	547	Cmab + CT	29.93	10.5
COIN[58]	3	279	Cmab + FOLFOX	14.9	8.5
COIN	3	523	Cmab + CAPOX	15.0	7.4
Souglakos *et al*[59]	2	167	Cmab + FOLFIRI	25.7	10.0
Souglakos *et al*[59]	2	166	Cmab + CAPIRI	27.5	8.9
CELIM[60,61]	3	56	Cmab + FOLFOX6	35.7	11.2
CELIM	3	55	Cmab + FOLFIRI	29.0	10.5
CECOG/ CORE1.2.001[62]	2	74	Cmab + FOLFOX6	17.4	8.6
CECOG/ CORE1.2.001	2	77	Cmab + FOLFIRI	18.9	8.3
CRYSTAL[63,64]	3	316 (WT)	Cmab + FOLFIRI	23.5	9.9
Nordic VII[65]	3	97 (WT)	Cmab + Nordic FLOX(bolus)	20.1	7.9
COIN[66]	3	362 (WT)	Cmab + CAPOX/ FOLFOX	17.0	8.6
Borner[67]	2	37	Cmab + CAPOX	20.5	7.2
OPUS[68,69]	2	82 (WT)	Cmab + FOLFOX4	18.3	7.2

OS: Overall survival; PFS: Progression-free survival; Cmab: Cetuximab; N: Number of patients analyzed in the study; CRC: Colorectal cancer; WT: Wild type; CT: Chemotherapy treatment; FOLFIRI: Leucovorin + 5-fluorouracil + irinotecan; FOLFOX: Leucovorin + 5-fluorouracil + oxaliplatin; CAPOX: Capecitabine + oxaliplatin; CAPIRI: Capecitabine + irinotecan; Nordic FLOX: 5-fluorouracil/leucovorin bolus + oxaliplatin.

decreasing Claspin, CDC45 and CDC6 levels expressed at the beginning of DNA replication[29]. Prewett *et al*[30] found that Cmab decreased the phosphorylation of ERK1/2 and AKT, resulting in the inhibition of ERCC1 and XPF. Additionally, Balin-Gauthier *et al*[29] reported that the mRNA level and protein expression of ERCC1 and XRCC1 declined after treatment with Cmab[31]. Last, EGFR expression in CRC cells increased when stimulated by oxa, which sensitized the treatment with Cmab[32,33]. By contrast, Cmab inhibited NOX1 expression, which assisted NADPH, as a coenzyme, to produce ROS. When the levels of ROS produced by the cell was reduced, the anti-tumor effect induced by oxa was also reduced[34,35].

It is known that oxa alone has little efficacy, and its activation requires fluoropyrimidine as a partner. Moreover, the toxicity of oxa-based chemotherapy and Cmab combination was different (Figure 2), varying with the mode of administration of fluoropyrimidine (infusional 5-Fu, bolus 5-Fu, and cap). Preclinical research studies addressing the question of the optimal administration method of 5-Fu are rare. One study showed that the longer the infusion time, the more significant the suppression of thymidylate synthase (TS)[11,36]. In that study, three different 5-Fu-sensitive human cancer cell lines, gastric cancer, colorectal cancer and breast cancer, were exposed to 5-Fu for either one hour or 24 h repeatedly. The 5-Fu concentration was fixed, and the two treatments had equivalent effective doses. The results showed that cells exposed to one-hour of 5-Fu developed resistance more rapidly than those exposed to 24 h of 5-Fu. Additionally, only a small fraction of one-hour exposed cells was cross-resistant to a 24-h treatment, whereas obvious cross-resistance was seen for 24-h exposed cells to a one-hour schedule. Moreover, increasing TS expression was observed in all of the 24-h exposed cells, but in only one cell line treated with one-hour 5-Fu. Hence, the author concluded that the effect of 5-Fu was determined by the mode of application because the inhibition of TS was more significant with a prolonged infusion time. Although the preclinical data are limited, the efficacy of 5-Fu infusion application has been demonstrated by clinicians. Aschele *et al*[37] suggested that the application schedule and biochemical modulators of 5-Fu-based chemotherapy determine the relationship between intratumoral TS levels and clinical outcomes.

Meanwhile, Cmab promotes 5-Fu activity by inhibiting TS[36,38]. Skvortsov *et al*[39] illustrated that in EGFR-overexpressed CRC cell lines, such as Caco-2, HRT-18, HT-29, WiDr and SW-480, TS expression were suppressed, whereas in the EGFR-negative cell line SW-620, inhibition disappeared. Additionally, the combined treatment of 5-Fu and Cmab was related to a synergistic activation of the MAPK pathway. The *in vitro* results were consistent with a meta-analysis showing that the efficacy of oxa and Cmab combination was optimized by infusional 5-Fu[40].

The efficacy and toxicity mechanisms of Cmab and iri-based chemotherapies are quite clear compared with those of the oxa and Cmab combination. According to clinical trial results, iri is the only cytotoxic agent combined with all targeted drugs that is recommended in the first-line treatment of CRC. The reciprocal interactions of Cmab and iri result in reduced DNA damage repair, increased SN-38 plasma concentration and enhanced suppression of the EGFR signaling pathway. Chu *et al*[41] found that the EGFR inhibitor could reduce SN-38 excretion by suppressing ABB1 *in vivo*. The researcher studied the influence of Cmab on the iri concentration and its effective metabolite SN-38 in mice *via* HPLC analysis. Human CRC xenografted nude mice were generated and treated with oral iri alone or with iri following pretreatment with Cmab. They found that the AUC of SN-38 in the plasma and tumors of mice given the combined treatment was nearly 1.7-fold higher than that in mice treated with iri alone, which demonstrated that Cmab was associated with the distribution of iri into tissues. In addition, Yashiro *et al*[42] suggested that EGFR inhibitors decreased the expression of uridinediphosphoglucuronate glucuronosyltransferase 1A1 (UGT1A1) and ABCG2 to prolong the active ingredient concentration. However, the improved

efficacy did not occur without toxicity. The common adverse events of the combination treatments include hand-foot syndrome, which occurs at a rate as high as 34.6%; diarrhea, which occurs at a rate of approximately 15%; and skin toxicity (Figure 3).

COMMON ADVERSE EVENTS AND SUGGESTED MANAGEMENT

Dermatologic

Of particular note, dermatologic toxicities have received considerable attention in clinical practice because of their prognostic role in Cmab treatment[23,43,44]. As the most common side effect related to anti-EGFR therapy, the incidence of all grades of rash is as high as 45%-95%, of which 5%-18% are grades 3 or above[43]. Papulopustular eruption, also known as acneiform rash, is the most common dermatologic adverse event induced by EGFR inhibitor treatment. In addition, nail changes, ocular changes, hair changes, pruritis, photosensitivity, xerosis and erythema also appear during Cmab treatment[44]. Usually, the rash occurs within two to three days following initiation of Cmab treatment, and it worsens within one to three weeks. Although not life threatening, the dermatologic toxicities are significantly related with impaired quality of life, especially in younger patients because of the discomfort and detriment in some obvious locations, such as the face[45,46].

Indeed, oral minocycline or doxycycline is suggested as a prophylactic treatment during Cmab treatment. In addition, broad-spectrum sunscreen should be applied to reduce sunshine exposure, and alcohol-containing skin products should be avoided. For dry skin, emollients and mild topical steroids, such as 1% hydrocortisone cream twice or three times a day, are suggested. For papulopustular eruptions, topical antibiotics should be administered. For moderate pruritus or tender skin rashes, 0.1% triamcinolone or 2.5% hydrocortisone cream is recommended. The Cmab treatment should be adjusted once a grade 3 rash appears, and oral corticosteroids or even oral antibiotics are administered to these patients.

Gastrointestinal/hepatobiliary

Gastrointestinal toxicities are common adverse events for traditional chemotherapy regimens and are also a common toxic effect of targeted therapies. The frequency of diarrhea and colitis of all grades is 20%-66%, and it is 2%-16% for grade 3 or above. In addition, 38%-43% of patients exhibit elevated transaminase elevation and 7% to 32% from mucositis/stomatitis[43]. The appearance of diarrhea is due to widespread mucosal inflammation, from oropharyngolaryngeal inflammation to frank stomatitis. It is reported that the mechanism of this diarrhea is associated with Notch signaling pathway inhibition, which results from the transformation of proliferative undifferentiated intestinal crypt cells into secretory goblet cells[47-49]. Regarding the elevated transaminase levels, this increase might be associated with the inhibition of UGT1A1, the polymorphic variants of which contribute to isolated hyperbilirubinemia in Gilbert's syndrome[50,51].

To treat diarrhea and colitis, the cause of diarrhea should be determined along with the administration of anti-motility agents, for example, loperamide and diphenoxylate/atropine, especially for patients who have received chemotherapy combined therapy. Alcohol- or peroxide-based mouthwashes should be avoided in the management of mucositis and stomatitis, and anesthetic mouthwashes should be administered at the same time. If infection is found, antifungal agents should be applied. Liver function laboratory investigations should be taken at baseline and at least once monthly if transaminase is elevated during treatment. If the aspartate aminotransferase (AST) or alanine aminotransferase (ALT) levels are above 53 upper limits of normal (ULN), treatment should be withheld. If the AST or ALT levels are less than 33 ULN, treatment can be resumed at a reduced dose.

Hypomagnesemia

Hypomagnesemia often occurs as a metabolic abnormality during Cmab treatment, and the frequency of all grades is 11%-38%, of which 4%-5% is grade 3 or 4[43]. A positive association has been demonstrated between total treatment duration and defective renal magnesium reabsorption, and the age and baseline serum magnesium concentrations are negatively associated with hypomagnesemia[52]. The activity and distribution of the transepithelial magnesium channel TRPM6 is regulated by EGF, resulting in excretion of renal magnesium. In addition, Thebault *et al*[53] and Groenestege *et al*[54] discovered an *EGFR* gene point mutation that contributes to isolated hypomagnesemia.

The suggested management is the optimal management of diarrhea. Significant QT-prolongation potential medications should be avoided. Oral supplementation should be used if necessary. Patients with grade 2 hypomagnesemia should be given a weekly intravenous infusion of replacement magnesium. Treatment should be initiated for patients with grade 3/4 or symptomatic hypomagnesemia, and intravenous magnesium should be increased to every 2-3 d.

Ocular

Corneal abnormalities (keratoconjunctivitis, corneal ulceration) and corneal epithelium are the direct ocular toxicity effects of Cmab, and Cmab indirectly affects the associated glands and appendages, resulting in indirect adverse effects (meibomitis, cicatricial ectropion, dry eye). The incidence of all-grade ocular toxicity is reported to be 4%-18%, of which less than

1% is above grade 3[43].

The advised management of ocular toxicity is to continue the treatment. Artificial tears are applied if necessary, and antibacterial ointment should be used if infection is confirmed. Ophthalmologic evaluation is recommended for patients with vision changes, persistent eye pain, photosensitivity or presence of other drug-induced ocular anomalies, such as trichiasis. For patients with grade 3 symptoms, treatment should be withheld.

CONCLUSION

The advent of EGFR mAbs, especially Cmab, has provided a meaningful transformation in the available treatment options for CRC. Nevertheless, although the application of these targeted drugs has yielded a tremendous benefit for patients with advanced CRC and although these drugs have even outperformed conventional chemotherapies, their efficacy is accompanied by some undesired complications. In addition, combination treatments comprising EGFR mAbs and traditional cytotoxic agents, such as 5-fluorouracil, oxa and iri, have resulted in a more complex management of CRC treatment-related side effects compared with EGFR mAb monotherapy. We believe that a thorough recognition of the toxicities from EGFR mAbs is essential for physicians to evaluate the potential risks into the correct context of clinical benefit. Therefore, it is imperative to understand and appropriately address the treatment dilemmas of cetuximab combined with chemotherapy for the management of metastatic CRC.

Currently, investigations of precise drug mechanisms are needed to refine existing drugs, to develop new targeted therapies and to optimize amenable therapeutic settings for personalized medicine. However, the existing research to precisely guide regimen and dose selection and to better reveal the mechanisms of interactions related to efficacy and toxicity is limited. Hence, more information is needed, especially for the reciprocal interactions between iri/oxa-based chemotherapies and mAbs. Additionally, a better understanding of the mechanisms of targeted agents regarding their activity, metabolism and resistance is also urgently needed to employ these combined therapies most effectively. Finally, an improved knowledge of robust markers of prognosis and toxicity is vital to implement treatment strategies and accurately select patients. Consequently, emerging molecular technologies will result in a definitive treatment for colorectal cancer.

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ORIGINAL ARTICLE

Basic Study

Tumor-specific expression of shVEGF and suicide gene as a novel strategy for esophageal cancer therapy

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Abstract

AIM: To develop a potent and safe gene therapy for esophageal cancer.

METHODS: An expression vector carrying fusion suicide gene (yCDglyTK) and shRNA against vascular endothelial growth factor (VEGF) was constructed and delivered into EC9706 esophageal cancer cells by calcium phosphate nanoparticles (CPNP). To achieve tumor selectivity, expression of the fusion suicide gene was driven by a tumor-specific human telomerase reverse transcriptase (hTERT) promoter. The biologic properties and therapeutic efficiency of the vector, in the presence of prodrug 5-fluorocytosine (5-FC), were evaluated *in vitro* and *in vivo*.

RESULTS: Both *in vitro* and *in vivo* testing showed that the expression vector was efficiently introduced by CPNP into tumor cells, leading to cellular expression of yCDglyTK and decreased VEGF level. With exposure to 5-FC, it exhibited strong anti-tumor effects against esophageal cancer. Combination of VEGF shRNA with the fusion suicide gene demonstrated strong anti-tumor activity.

CONCLUSION: The shVEGF-hTERT-yCDglyTK/5-FC system provided a novel approach for esophageal cancer-targeted gene therapy.

Key words: Esophageal cancer; Suicide gene; RNA interference; Vascular endothelial growth factor; Nanoparticles

Core tip: Esophageal cancer is a highly aggressive neoplasm with poor prognosis and low survival rates. In this study, an expression vector carrying a fusion suicide gene (yCDglyTK) and shRNA against vascular endothelial growth factor (VEGF) was constructed and delivered into EC9706 esophageal cancer cells by calcium phosphate nanoparticles (CPNP). To achieve tumor selectivity, the expression of the fusion suicide gene was driven by a tumor-specific human telomerase reverse transcriptase promoter. Our results showed that the novel expression vector was efficiently introduced into EC9706 cells by CPNP, leading to cellular expression of yCDglyTK and decreased VEGF level. With exposure to 5-fluorocytosine, it exhibited strong anti-tumor effects against esophageal cancer both *in vitro* and *in vivo*. Combination of VEGF shRNA with the fusion suicide gene demonstrated strong anti-cancer effects. Our study provides a novel approach for esophageal cancer-targeted gene therapy.

Liu T, Wu HJ, Liang Y, Liang XJ, Huang HC, Zhao YZ, Liao QC, Chen YQ, Leng AM, Yuan WJ, Zhang GY, Peng J, Chen YH. Tumor-specific expression of shVEGF and suicide gene as a novel strategy for esophageal cancer therapy. *World J Gastroenterol* 2016; 22(23): 5342-5352 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i23/5342.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i23.5342

INTRODUCTION

Esophageal cancer is one of the most prevalent cancers in the world. It has high incidence and mortality rates, especially in Africa, East Asia, and North America[1]. Histologically, the most common type of esophageal cancer is esophageal squamous cell cancer (ESCC), which accounts for more than 90% of all cases[2]. Despite advances in diagnosis and treatment of ESCC during recent years, postoperative survival rates have not improved in the last decade. The 5-year overall survival rate is still less than 20%[3,4]. Novel and effective treatments are urgently needed for esophageal cancer.

Gene therapy has been recognized as a promising strategy for cancer treatment[5]. Among various gene therapy approaches, suicide gene therapy holds great promise since suicide gene expression can be manipulated to a particular tissue[6]. It transfers prodrug-activating enzyme genes into cancer cells. These genes encode enzymes that can catalyze non-toxic prodrugs into cytotoxic agents to confer drug sensitivity to certain types of cells[7]. Cytosine deaminase (CD) and herpes simplex type I thymidine kinase are the two most intensively studied suicide genes. Previous studies showed that a fusion gene (CDglyTK) of yeast cytosine deaminase (yCD) and thymidine kinase (TK) was more effective than either single suicide gene[8]. In addition, suicide gene expressed in localized areas of tumor can produce high local concentration of cytotoxic agent [*e.g.*, 5-fluorouracil (5-FU)], thus killing the neighboring tumor cells[9]. Expression of yCDglyTK, together with the treatment of prodrug 5-fluorocytosine (5-FC) exhibited targeted therapeutic effects[10-12].

A major limitation for the clinical use of suicide gene therapy for cancer is its proneness to cause side effects due to a lack of tumor specificity. The tissue-specific expression of suicide genes in tumor cells could be achieved by taking advantage of certain tumor-specific transcription regulatory elements, such as promoters and enhancers[13]. A number of tumor-specific promoters were employed in previous studies[14,15], and among them, the human telomerase reverse transcriptase (hTERT) promoter demonstrated the greatest tumor specificity[10]. The hTERT promoter is inactive in normal somatic cells[16,17] and may enable tumor-selective pharmaceutical effects of therapeutic genes.

Angiogenesis plays a vital role in the process of growth and metastasis of solid tumors[18]. Vascular endothelial growth factor (VEGF) has been found to be an important angiogenesis factor in cancer development[19]. It exerts various biological effects on the growth and spread of tumors, including induction of proteinases, cell mitogenesis, cell migration, increase of vascular permeability, and survival maintenance of newly formed blood vessels. Overexpression of VEGF

has been found in about 60% of esophageal carcinoma cases[20,21]. Downregulating VEGF may be a potential targeted treatment strategy for esophageal cancer[22].

Another key consideration for developing a successful gene therapy system is improving transfection efficiency while minimizing toxicity and enhancing stability[23,24]. A number of viral vectors and non-viral plasmids have been developed for gene therapy. However, viral vectors have some serious drawbacks, such as triggering immune response[25], severe hepatic inflammation[26], random chromosomal integration[27], and cytotoxicity to host cells[28]. Non-viral vectors are considered more promising as gene delivery vehicles because they are safe, easy to synthesize, cost-effective, and have a low degree of immunogenicity[29]. In our previous work, we developed the calcium phosphate nanoparticle (CPNP) as a novel non-viral tool for efficient gene delivery[11,12,30]. CPNP-delivered suicide genes mediated tumor specific cytotoxicity in gastric cancer and colon cancer[11,12].

In this study, tumor-specific shVEGF-yCDglyTK expression cassette was delivered using CPNP into human esophageal cancer cells and xenograft esophageal carcinoma. The therapeutic efficacy of this novel gene therapy system was evaluated, and the results suggest it was specific and effective for esophageal carcinoma.

MATERIALS AND METHODS

Cell lines and cell culture

Human esophageal squamous cell cancer cell line EC9706 and normal human lung fibroblast cell line HLF were obtained from the Central Laboratory of Xiangya Hospital, Central South University. A human cervical adenocarcinoma cell line HeLa was acquired from the Cancer Research Institute, Central South University. Cells were cultured in Roswell Park Memorial Institute 1640 medium (Hyclone, Logan, UT, United States) supplemented with 10% heat-inactivated fetal bovine serum and maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37 ℃.

Construction of tumor-specific shVEGF-yCDglyTK expression cassette

Human hTERT promoter was amplified by polymerase chain reaction (PCR) from HeLa genomic DNA by using the following primers: forward primer 5′-GCGACGCGTGATTCGCGGGCACAGACG-3′and reverse primer 5′- AAACTCGAGCCACGTGCGCAGCAGGAC-3′. The product was cloned into the Mlu I and Xho I sites of the pGL3-Basic vector. pcDNA3.1(-)-CV-yCDglyTK[11] and pGenesil-shVEGF[31] were constructed in previous studies. pcDNA3.1(-) hTERT-yCDglyTK was made by replacing the carcinoembryonic antigen (CEA) promoter with the hTERT promoter. An expression cassette pcDNA3.1(-)-shVEGF-hTERT-yCDglyTK, which expressed shVEGF and yCDglyTK, was constructed as shown in Supplementary Figure 1. All constructs were confirmed by sequencing.

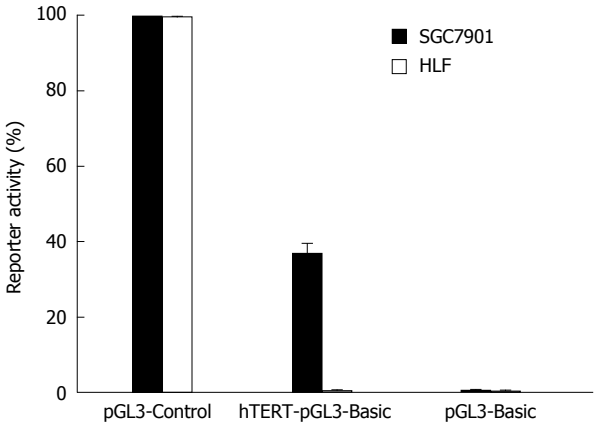

Figure 1 Human telomerase reverse transcriptase promoter activities in cancerous and normal cells. EC9706 and HLF cells were transfected with hTERT reporter vector (hTERT-pGL3 basic). pGL3-Control and pGL3 basic were transfected as positive and negative controls, respectively. hTERT: Human telomerase reverse transcriptase.

Transcriptional activity analysis of the hTERT Promoter

EC9706 and HLF cells were seeded in 24-well plates with 1 × 10^5 cells per well. Three plasmids, hTERT-pGL3 Basic, pGL3-Basic, and pGL3-control, were transfected when the cell monolayer reached 80%-85% confluence. The activities of luciferase and beta-galactosidase (β-gal) were detected 48 h post transfection. The luciferase activity was normalized against β-gal activity and expressed as relative luciferase activity.

Transfection efficiency of CPNP-DNA complexes

CPNP was produced according to previous studies[11,12,30]. The characteristics of CPNP have been described previously[11]. Two micrograms of pEGFP-N1 DNA were mixed with 20 μg of CPNP to generate the CPNP-pEGFP-N1 complexes. EC9706 cells were seeded in 12-well plates at a density of 2 × 10^5 cells per well. When the cell monolayer reached 80%-85% confluence, the CPNP-pEGFP-N1 complexes were added to the cells. The pEGFP-N1 DNA-liposome complexes (10 μg:2 μg) were used as a positive control. Transient transfection efficiency after 48 h was determined by examining GFP expression using flow cytometry and fluorescence microscopy.

Stable transfection

EC9706 cells were seeded in 6-well plates at a density of 2 × 10^5 cells each well. When the cell reached 70%-80% of confluence, pcDNA3.1(-) null, pGenesil-shVEGF, pcDNA3.1(-)-hTERT-yCDglyTK, and pcDNA3.1(-)-hTERT -shVEGF-yCDglyTK were mixed with CPNP (2 μg of DNA: 20 μg of CPNP), respectively[30]. At 48 h after transfection, G418 was added to cell culture medium, the final concentration

of which was 600 μg/mL. The G418-resistant colonies were picked 16 d later and kept in 96-well plates. These cells were maintained in selective medium containing 200 μg/mL of G418.

Reverse transcription-PCR and Western blot analysis

Total RNA from parental EC9706 cells and stable transfected cells was prepared by using TRIzol reagent (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's protocol. Reverse transcription (RT)-PCR of yCDglyTK and VEGF were carried out with AMV reverse transcription kit (Promega, Madison, WI, United States). Primers for yCDglyTK were: forward primer 5'-GGGAGATTAGAGGGCAAAGTGT-3', reverse primer5'-ACGGCGTCGGTCACGGCATAA-3'. The yCDglyTK PCR product was 707 bp. A VEGF PCR product of 112 bp was produced by forward primer 5'-TCTTCAAGCCATCCTGTGTG-3'and reverse primer 5'-ATCCGCATAATCTGCATGGT-3'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control, and a PCR product of 101 bp was produced by forward primer 5'-CCTGTTCGACAGTCA GCCG-3'; reverse primer 5'-CGACCAAATCCGTTGACTCC-3'. The amplified fragments were separated in 2% agarose gels for visualization.

For Western blot assay, 30 μg protein from each sample was loaded for 10% sodium dodecyl sulfate gel electrophoresis and transferred to polyvinylidene fluoride membrane. The membranes were blocked with 5% (w/v) non-fat dry milk in Tris-buffered saline with tween (TBST) buffer at room temperature for 1 h. The membranes were then incubated overnight at 4℃ with anti-CD antibody (QED Bioscience Inc., San Diego, CA, United States). After washing three times with TBST buffer, blots were incubated for 2 h at room temperature with horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody (Sigma, St. Louis, MO, United States). Signals were detected by enhanced chemiluminescence with Western blot detection system (Amersham, Piscataway, NJ, United States). GAPDH was used as a control to ensure equal protein loading.

Cytotoxicity assay

Parental and stable EC9706 cells were seeded at a density of 5000 cells per well in 96-well plates and incubated at 37℃ for 24 h. 5-FC was added into the culture medium, and the final concentration was 200 μg/mL. Methylthiazole tetrazolium (MTT) assays were conducted after incubation for 24, 48, 72, and 96 h, respectively. At the end of the incubation period, 20 μL of MTT stock solution (5 mg/mL, Sigma) was added per well, and the supernatant was carefully removed 4 h later. The formazan crystals were dissolved in dimethyl sulfoxide (DMSO, Promega). Optical density (OD) was determined by using a multi-well plate reader by measuring absorbance at 570 nm with a 690 nm reference wavelength. The background absorbance of medium was subtracted. All samples were assayed in triplicate, and the mean value for each experiment was analyzed. Cell growth curves were plotted with culture time on the horizontal axis and OD570 values on the vertical axis.

Hoechst staining

Nuclear morphology changes of apoptotic cells were detected by staining with Hoechst 33258. Parental EC9706 cells and stable cells transfected with empty vector, shVEGF, yCDglyTK, and shVEGF-yCDglyTK were cultured in complete medium supplemented with 5-FC (200 μg/mL). After 48 h, cells were washed three times with phosphate buffered saline (PBS) and stained with 5 μg/mL Hoechst 33258 for 30 min in the dark. Stained nuclei were visualized using a fluorescence microscope with a wavelength of excitation at 355 to 366 nm.

Flow cytometry analysis of cell apoptosis

Flow cytometry was performed as previously described[11]. EC9706 cells stably expressing shVEGF, yCDglyTK, and shVEGF-yCDglyTK and untransfected EC9706 cells were seeded in 10 cm dishes, respectively. 5-FC was added to the culture medium at a concentration of 200 μg/mL when the cells reached 90%-95% confluence. Cells were pelleted after 48 h, washed with PBS, and then resuspended in staining buffer (HEPES supplemented with 2.5 mmol/L $CaCl_2$). Flow cytometry was employed for detecting cell apoptosis after addition of fluorescein isothiocyanate-labeled annexin V and incubation for 15 min at 4℃.

Animal experiments

The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Central South University. Twenty-five female BALB/c nude mice (4-6 wk old, 18-20 g) were acquired from Slac Laboratory Animal of Shanghai Co. Ltd, China. A suspension of 5×10^6 EC9706 cells was inoculated subcutaneously into the right flank of nude mice. After 10 d, when transplanted tumors reached a volume of 100-200 mm^3, the mice were randomized into five groups, named A, B, C, D, and E, with five mice in each group. Group A was the non-treating control group; Group B received intratumoral injection of the CPNPs/pcDNA3.1(-)null complex; Group C received intratumoral injection of the CPNPs/pGenesil-shVEGF complex; Group D received intratumoral injection of the CPNPs/pcDNA3.1(-)-hTERT-yCDglyTK complex; and Group E received intratumoral injection of the CPNPs/pcDNA3.1(-)shVEGF-hTERT -yCDglyTK complex.

The intratumoral injections of CPNP/DNA complexes were carried out every other day and repeated three times in total. One day after the first intratumoral injection, mice in groups B, D, and E received daily

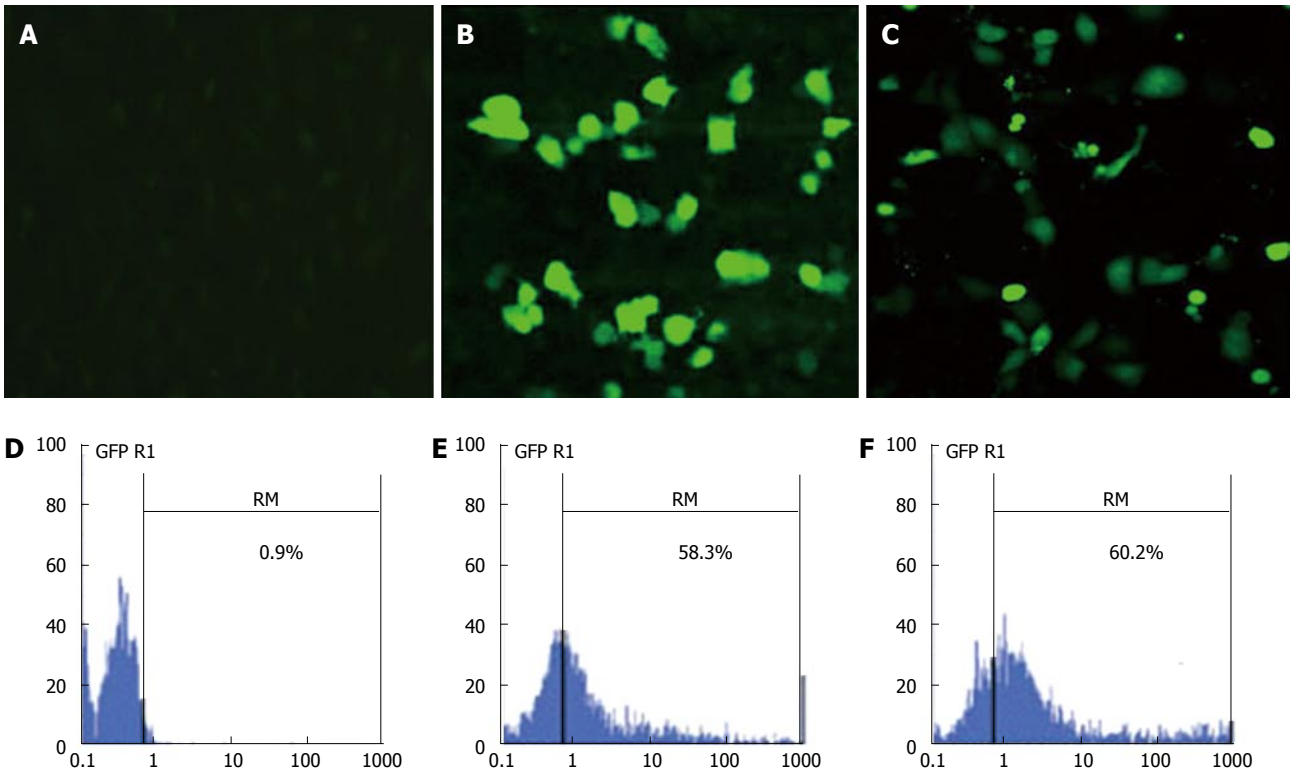

Figure 2 Transfection efficiency of calcium phosphate nanoparticles. A-C: Images of transfected EC9706 cells acquired by fluorescence microscope at magnification × 200 after 48 h of transfection. Negative control (A), CPNP-GFP complex (B), Liposome/GFP complex (C); D-F: Qualitative analysis of transfection efficiency by flow cytometric assay. Negative control (D), CPNP-GFP complex (E), Liposome/GFP complex (F). CPNP: Calcium phosphate nanoparticles; GFP: Green fluorescent protein.

intraperitoneal injection of 5-FC (500 mg/kg) for 14 consecutive days. The body weights of mice were recorded twice every week, and symptoms of side effects including change in behavior and food withdrawal were closely monitored. The longest (L) and shortest (W) perpendicular tumor diameters were measured every 3 d with calipers. To estimate the tumor volume; the following formula: $V = (1/2)W^2 \times L$ was used to calculate the three-dimensional volume of the xenograft. The growth curve was drawn according to the tumor volume. Animals were euthanized if the tumor diameter reached 1.5 cm or weight loss was more than 20% during the experiment. All surviving mice were euthanized by CO_2 inhalation and cervical dislocation at the end of the experiment.

Immunohistochemistry assays

The esophagus cancer xenograft tissue was cut at 4-μm thickness. The sections were incubated overnight at 4 ℃ with primary polyclonal antibody (anti-CD). The anti-rabbit antibody (Abgent, San Diego, CA, United States) was used as secondary antibody and incubated with sections for 30 min at 37 ℃. Color development was performed with the streptavidin-peroxidase system (Sigma-Aldrich). The chromogen was 3,3-diaminobenzidine tetrahydrochloride. Nuclei were lightly counterstained with hematoxylin. Microvessel counts (MVC) were analyzed to evaluate the degree of angiogenesis. Sections were immunostained with anti-CD34 antibody and examined at 200 × magnification. At least three microscopic images were collected and further analyzed with Axion Vision Rel 4.6 software to obtain MVC.

RESULTS

Assembly of tumor-specific shVEGF-yCDglyTK expression vector

In order to develop a more effective treatment for esophageal cancer, a novel expression plasmid, which could express a shRNA for VEGF and a fusion suicide gene yCDglyTK (Supplementary Figure 1), was constructed. The expression of shVEGF was under the control of the U6 promoter, a pol Ⅲ promoter; the expression of yCDglyTK was controlled by the tumor-specific hTERT promoter.

Specific transcriptional activities of the hTERT promoter in esophageal carcinoma cells

Activities of the hTERT promoter were determined by luciferase assay in EC9706 esophageal cancer cells and HLF normal lung fibroblast cells. The pGL3-Control vector containing SV40 promoter and enhancer sequences was used as a positive control. Luciferase activity driven by the hTERT promoter was much higher in EC9706 cells than in the HLF cells (Figure

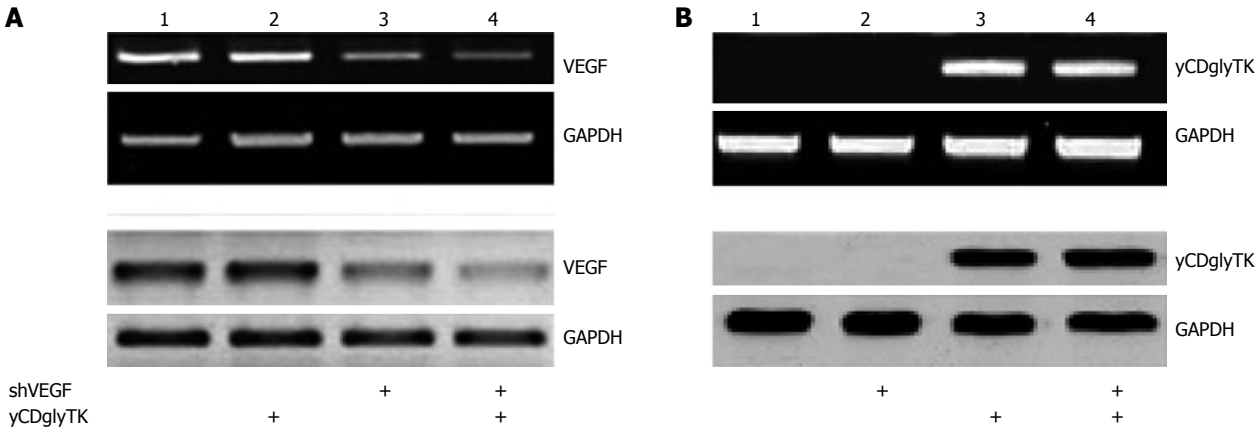

Figure 3 Changes of vascular endothelial growth factor and yCDglyTK expression in established stable cell lines. A: Representative VEGF mRNA and protein expression were analyzed by RT-PCR (top panel) and western blot (bottom panel), respectively. GAPDH was used as an internal control. Lane 1, EC9706/null; lane 2, EC9706/yCDglyTK; lane 3, EC9706/shVEGF; lane 4, EC9706/shVEGF-yCDglyTK; B: Representative yCDglyTK mRNA and protein expression were analyzed by semiquantitative RT-PCR (top panel) and western blot (bottom panel), respectively. GAPDH was used as an internal control. Lane 1, EC9706/null; lane 2, EC9706/shVEGF; lane 3, EC9706/yCDglyTK; lane 4, EC9706/shVEGF-yCDglyTK. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; RT-PCR: Reverse transcription polymerase chain reaction; VEGF: Vascular endothelial growth factor.

1). The results indicated that tumor specificity driven by the hTERT promoter could be achieved in EC9706 esophageal carcinoma cells.

Transfection efficiency of CPNP on esophageal cancer cells

EC9706 cells were transiently transfected with CPNP/green fluorescent protein (GFP) complex to evaluate the transfection efficiency of CPNP. EC9706 cells were assessed 48 h after transfection for GFP expression by using fluorescence microscopy and flow cytometry analysis. As shown in Figure 2, the proportion of GFP expressing cells transfected with CPNP was 58.3%, similar to that in liposome-medicated transfection group (60.2%). These data suggested that CPNP is an efficient strategy to deliver DNA into EC9706 esophageal cancer cells.

MTT assay was carried out to test the toxicity. Compared to the control group, liposome inhibited the cell growth by about 21%, while CPNP-DNA group had little effect on the cell growth (Supplementary Figure 1).

Changes in expression of VEGF and yCDglyTK by introducing the expression construct

RT-PCR and western blot were used to determine the expression of VEGF and yCDglyTK. Compared with cells transfected with pcDNA3.1(-), EC9706 cells transfected with pcDNA3.1(-)-shVEGF-hTERT-yCDglyTK showed significantly decreased expression of VEGF at both the mRNA and protein levels. No significant changes of VEGF expression were observed in EC9706 cells transfected with pGenesil-shVEGF and pcDNA3.1(-)-shVEGF- hTERT-yCDglyTK (Figure 3A). yCDglyTK expression was not present in cells transfected with pcDNA3.1(-). Its expression was substantially increased at both the mRNA and protein level by introduction of pcDNA3.1(-) -shVEGF-hTERT-yCDglyTK into EC9706 cells (Figure 3B).

Effects of shVEGF-yCDglyTK/5-FC system on cell growth and apoptosis in EC9706 cells

MTT assay was performed to assess cytotoxicity of the shVEGF-yCDglyTK/prodrug system in EC9706 cells. Parental and stable EC9706 cells were treated with 200 μg/mL of 5-FC. Cells were harvested at 24, 48, 72, and 96 h after 5-FC administration and subjected to MTT assay. As shown in Figure 4A, 96 h after 5-FC treatment, cells growth in the shVEGF group, yCDglyTK group, and shVEGF-yCDglyTK group was inhibited by 36%, 85%, and 94%, respectively.

Forty eight hours after 5-FC exposure, cell apoptosis were determined by Hoechst staining and flow cytometry, respectively. Hoechst staining showed that 5-FC induced a significant increase in apoptosis in EC9706 cells stably modified with the pcDNA3.1(-)-shVEGF-hTERT-yCDglyTK (Figure 4B). In contrast, apoptotic cells were barely found in parental EC9706 cells and cells carrying empty vector. Consistent results were found by the flow cytometry analysis (Figure 4C). Apoptotic population (early and late apoptotic) in EC9706 cells expressing shVEGF, yCDglyTK, and shVEGF together with yCDglyTK were 24.96%, 48.06%, and 62.9%, respectively. The percentage of apoptotic cells in untransfected EC9706 cells and EC9706 null cells transfected with an empty vector was only 0.97% and 1.3%, respectively. These results showed that both shVEGF and yCDglyTK expression could induce apoptosis and that combining them had the strongest effect on apoptosis induction.

Anti-tumor activity of shVEGF-yCDglyTK/5-FC system in vivo

The anti-tumor activity of the shVEGF-yCDglyTK/5-FC system was investigated with EC9706 xenograft

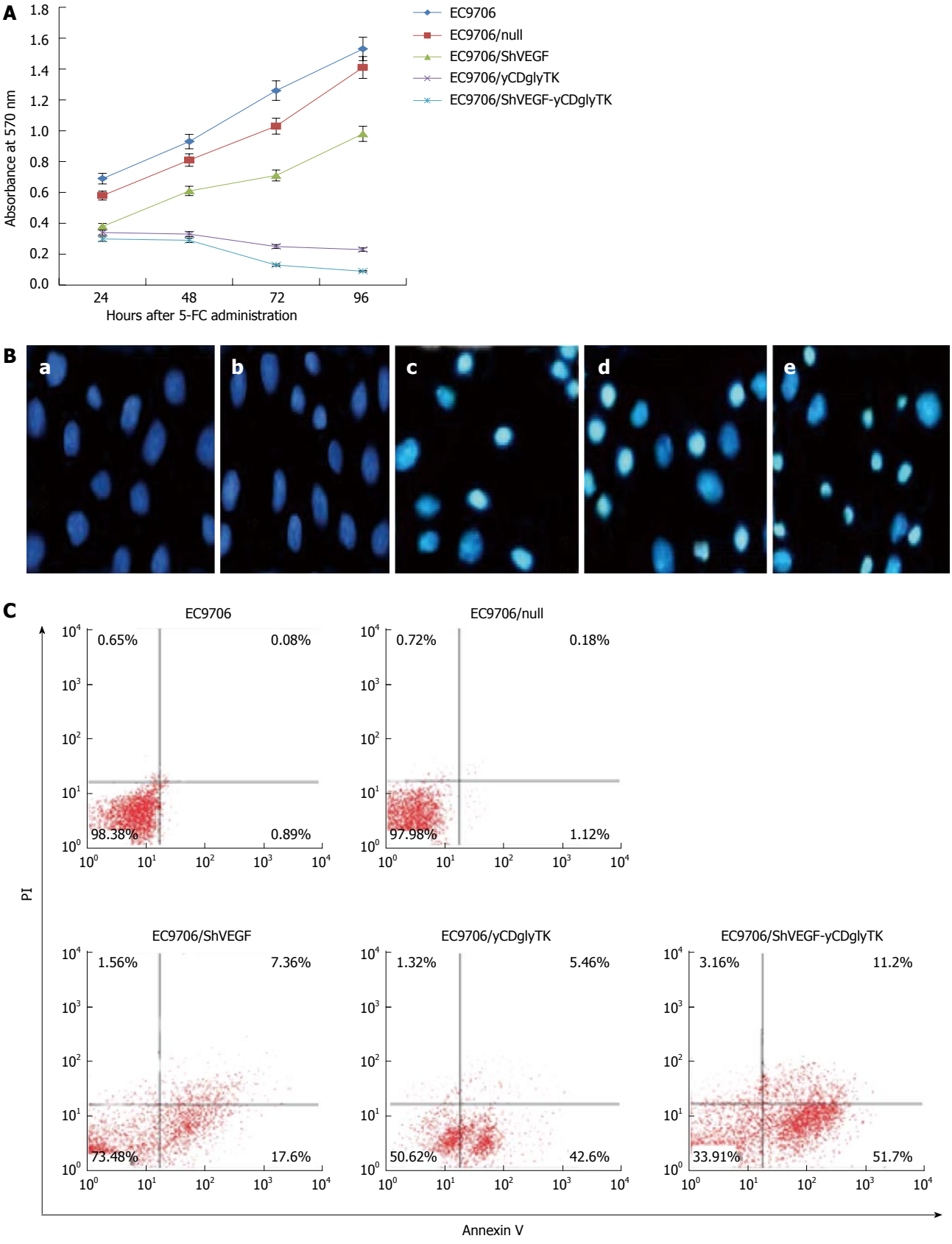

Figure 4 Effects of shVEGF-yCDglyTK/5-FC system on EC9706 cells. A: Cell viability of parental and stable EC9706 cells were determined with MTT assay at various time points after 5-FC treatment. The results shown are representative of three independent experiments; B: Representative images of Hoechst 33258-stained nuclei at magnification × 200. Apoptotic nuclei are condensed or fragmented; parental EC9706 cells (a); EC9706/null (b); EC9706/shVEGF (c); EC9706/yCDglyTK (d); EC9706/shVEGF-yCDglyTK (e); C: Representative dot plots of flow cytometry analysis. The numbers represent the percentage (%) of cells. Upper right quadrant, late apoptosis; lower right quadrant, early apoptosis; lower left, live cells. 5-FC: 5-fluorocytosine.

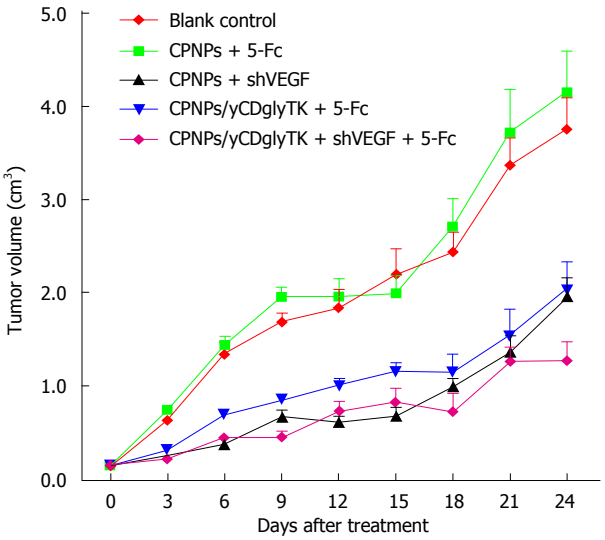

Figure 5 shVEGF-yCDglyTK/5-FC system inhibited tumor growth in the EC9706 xenograft model. Twenty-five BALB/C nude mice bearing EC9706 xenografts were randomized into five groups. The CPNPs/null, CPNPs/shVEGF, CPNPs/yCDglyTK or CPNPs/shVEGF-yCDglyTK complexes were delivered by intratumoral injection every other day, and the injection was repeated three times in total. 5-FC (500 mg/kg) was administered daily for 14 consecutive days. Tumors were measured every 3 d. All of the mice were scarified at day 36 after inoculation.

models. Tumors receiving CPNPs/null injection grew at a similar rate to that of the blank control group. On day 24, when compared with blank control group, CPNPs/yCDglyTK + 5-FC group and CPNPs + shVEGF group showed about 46% and 48% tumor growth inhibition. When combining CPNPs/yCDglyTK + 5-FC and shVEGF, the tumor growth was inhibited by 66% at day 24. These results demonstrated the potential benefit of the shVEGF-yCDglyTK/5-FC system for treating esophageal cancer (Figure 5).

Immunohistochemistry staining for yCDglyTK and VEGF

The expression and distribution of yCDglyTK and VEGF in the tumor xenograft were visualized by using immunohistochemistry staining. As shown in Figure 6A, tumor tissue expression of yCDglyTK was detected in mice injected with CPNPs/yCDglyTK or CPNPs/shVEGF-yCDglyTK. Meanwhile, it was not present in other groups, including non-treatment, sham control, and CPNPs/shVEGF groups. VEGF expression was quantified and represented by integrated optical density (IOD) values (Figure 6B). VEGF levels were significantly decreased in tumor tissues receiving injection of CPNPs/shVEGF or CPNPs/shVEGF-yCDglyTK, when compared with those of other groups ($P < 0.01$). No significant difference in VEGF expression was found between these two groups ($P > 0.05$). Since VEGF is an important angiogenesis factor, neovascularization was assessed by quantification of MVD. CPNPs/shVEGF and CPNPs/shVEGF-yCDglyTK groups showed lower MVD as compared to other groups (Figure 6C). The results suggested that the intratumoral injection of CPNPs/shVEGF-yCDglyTK could effectively inhibit neovascularization by down-regulating VEGF expression.

DISCUSSION

Esophageal carcinoma is a common cause of death globally. Traditional remedies for esophageal cancer, such as surgery, chemotherapy, and radiotherapy, all have drawbacks. For example, surgery is applicable only if the tumor is diagnosed at an early stage. Chemotherapy and radiotherapy have serious side effects as a result of lacking tumor specificity. Therefore, novel methods are required for the treatment of esophageal cancer. Gene therapy, especially suicide gene therapy, has been studied extensively for cancer treatment[32]. For suicide gene therapy, the therapeutic transgenes can convert a non-toxic pro-drug, which easily penetrates the tumor cell membrane, into a cytotoxic drug[33]. However, present suicide gene therapies have limited success due to lack of tumor specificity and an effective gene delivery tool.

One key point for successful gene therapy is the development of a safe and effective gene delivery system. Viral vectors are the most widely investigated delivering system because of their high transfection efficiency. However, viral vectors have some serious drawbacks, such as triggering immune response, severe hepatic inflammation, and random chromosomal integration[25-27]. Among non-viral vectors, cationic lipids could cause toxic effects when repeatedly used and induce potent anti-inflammatory activity *in vivo*; calcium phosphate precipitation had low transfection efficiency. The use of nanoparticles has become one of the most promising vectors because of their high transfection efficiency and low toxicity. In previous studies, we developed a new gene delivery system using either CPNP or calcium carbonate nanoparticles (CCNP)[11,12,30,31,34]. In this study, CPNP successfully delivered the suicide gene into EC9706 esophageal cells, with low toxicity.

Inserting a tumor-specific promoter upstream of suicide genes has been proven to be a successful strategy to achieve targeted expression of suicide genes in tumor tissues. Previously, we successfully used CEA promoter for targeting gastric cancer and colon cancer[11,12]. In this study, the specificity against esophageal cancer cells was achieved using the hTERT promoter.

VEGF stimulates tumor cell proliferation and angiogenesis in tumor tissue, and overexpression of VEGF has been found in the majority of human cancers[35,36]. Silencing VEGF with RNA interference (RNAi) could inhibit tumor growth and metastasis[37,38]. In this study, a novel fusion gene vector carrying a suicide gene (yCDglyTK) and a VEGF shRNA was developed. This expression vector was transfected into EC9706 esophageal cells by CPNP. Increased

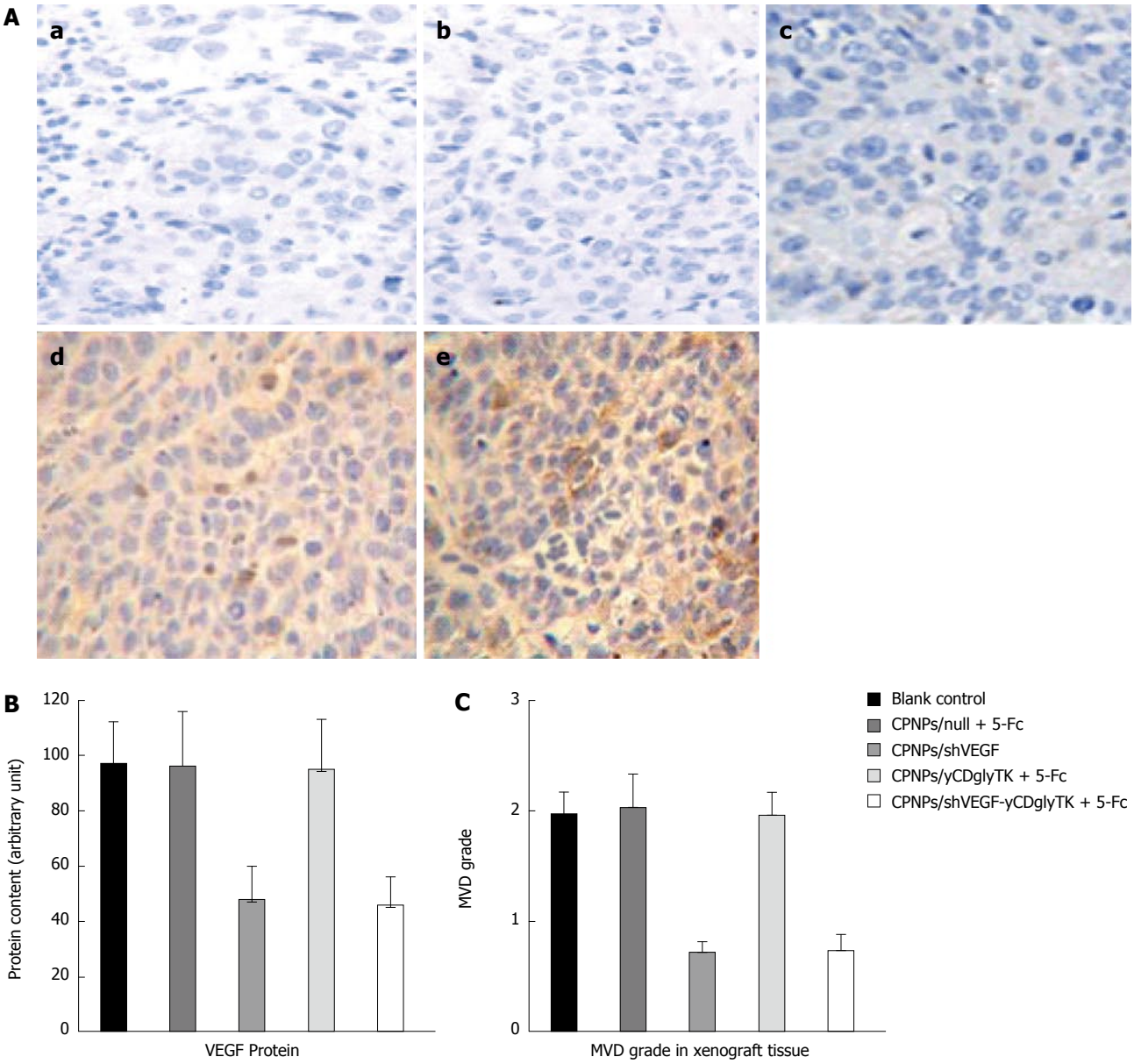

Figure 6 Immunohistochemistry analysis for yCDglyTK and vascular endothelial growth factor in EC9706 xenograft sections. A: Histological expression and distribution of yCDglyTK at magnification × 200; no-treatment control group (a); CPNPs/null + 5-FC (b); CPNPs/shVEGFX (c); CPNPs/yCDglyTK+5-FC (d); CPNPs/shVEGF-yCDglyTK +5-FC (e); B: Integrated optical density (IOD) values of VEGF expression in EC9706 xenografts. Anti-VEGF antibody was used for immunohistochemistry assay; C: Quantification of angiogenesis by microvessel counts (MVC) in EC9706 xenografts. Anti-CD34 was used for microvessel staining. CPNPs: Calcium phosphate nanoparticles; VEGF: Vascular endothelial growth factor; 5-FC: 5-fluorocytosine.

expression of yCDglyTK and decreased expression of VEGF were confirmed at both the mRNA and protein level in EC9706 cells.

In vitro antitumor activity of this novel system in the presence of prodrug was tested by MTT assay, Hoechst staining, and flow cytometry. The antitumor effect of the CPNP/shVEGF-yCDglyTK/5-FC system was further evaluated *in vivo* by using EC9706 cell xenograft model. Both yCDglyTK/5-FC and shVEGF successfully inhibited the tumor growth, and the combination of the two showed the strongest anti-tumor activity. Subsequent immunohistochemistry staining confirmed the expression of yCDglyTK, the knockdown of VEGF, and decreased neovascularization in the cancer tissue.

In summary, the CPNP/shVEGF-hTERT-yCDglyTK/5-FC system developed in this study may overcome several challenges for cancer gene therapy. The use of CPNP increased delivery efficiency, and insertion of hTERT promoter improved tumor specificity. VEGF-targeted shRNA further enhanced the anti-tumor effect. This combination of gene therapies exerted more potent anti-esophageal cancer activity *in vitro* and *in vivo*. The present study provides a novel and promising strategy for esophageal cancer treatment. We will try to optimize this approach to make it more feasible in the future clinical trials.

COMMENTS

Background

Esophageal cancer is one of the most prevalent cancers in the world. It has high incidence and mortality rates. Novel and effective treatment options are urgently needed for esophageal cancer.

Research frontiers

In recent years, gene therapy, especially suicide gene therapy, has been recognized as a promising strategy for cancer treatment.

Innovations and breakthroughs

In this study, tumor-specific shVEGF-yCDglyTK expression cassette was delivered using calcium phosphate nanoparticle (CPNP) into human esophageal cancer cells. The therapeutic efficacy of this novel gene therapy system was evaluated *in vitro* and *in vivo*, and results showed that it was an effective strategy for esophageal carcinoma treatment.

Applications

The present study provides a novel and promising strategy for esophageal cancer treatment.

Terminology

CPNP is a novel non-viral tool for efficient gene delivery.

Peer-review

This study investigated a novel technique for targeted gene therapy in an esophageal cancer model. The authors report transfection rates and altered gene expression profiles. The study is well conducted and well described. The techniques are appropriate and the experiments are clearly written. The study topic is interesting and relevant.

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ORIGINAL ARTICLE

Basic Study

Long-pulse gastric electrical stimulation protects interstitial cells of Cajal in diabetic rats *via* IGF-1 signaling pathway

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Author contributions: Liu S conceived and designed the experiments; Li H and Chen Y performed the experiments; Li H analyzed the data; Liu S and Hou XH contributed reagents/materials/analytical tools; Li H wrote the paper; and Hou XH is the main controller of the laboratory.

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Institutional animal care and use committee statement: All experiments involving animals were reviewed and approved by the Animal Care and Use Committee of Tongji Medical College, Huazhong University of Science and Technology (IACUC No. 488).

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Abstract

AIM: To investigate the effects of different parameters of gastric electrical stimulation (GES) on interstitial cells of Cajal (ICCs) and changes in the insulin-like growth factor 1 (IGF-1) signal pathway in streptozotocin-induced diabetic rats.

METHODS: Male rats were randomized into control, diabetic (DM), diabetic with sham GES (DM + SGES), diabetic with GES1 (5.5 cpm, 100 ms, 4 mA) (DM + GES1), diabetic with GES2 (5.5 cpm, 300 ms, 4 mA) (DM + GES2) and diabetic with GES3 (5.5 cpm, 550 ms, 2 mA) (DM + GES3) groups. The expression levels of c-kit, M-SCF and IGF-1 receptors were evaluated in the gastric antrum using Western blot analysis. The distribution of ICCs was observed using immunolabeling for c-kit, while smooth muscle cells and IGF-1 receptors were identified using α-SMA and IGF-1R antibodies. Serum level of IGF-1 was tested using enzyme-linked immunosorbent assay.

RESULTS: Gastric emptying was delayed in the DM group but improved in all GES groups, especially in the GES2 group. The expression levels of c-kit, M-SCF and IGF-1R were decreased in the DM group but increased in all GES groups. More ICCs (c-kit$^+$) and smooth muscle cells (α-SMA$^+$/IGF-1R$^+$) were observed in all GES groups than in the DM group. The average level

of IGF-1 in the DM group was markedly decreased, but it was up-regulated in all GES groups, especially in the GES2 group.

CONCLUSION: The results suggest that long-pulse GES promotes the regeneration of ICCs. The IGF-1 signaling pathway might be involved in the mechanism underlying this process, which results in improved gastric emptying.

Key words: Long-pulse gastric electrical stimulation; Interstitial cells of Cajal; Gastric emptying; IGF-1 pathway; Diabetes mellitus; Gastrointestinal motility disorders

Core tip: Gastric electrical stimulation (GES) regulates gastric motility and protects the interstitial cells of Cajal (ICCs). However, the mechanisms underlying these processes have not been determined. In this study, we report that long-pulse GES with three different parameters protected ICCs and that the insulin-like growth factor 1 signaling pathway is probably involved in this process. One GES parameter (5.5 cpm, 300 ms, 4 mA) showed immense potential as a clinical application for applying long-pulse GES as the optimal parameter.

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INTRODUCTION

Gastrointestinal motility disorders (such as early satiety, bloating and vomiting) often plague patients with long-standing diabetes. However, the pathogenesis of these disorders remains unclear, and effective pharmacological management is limited for these symptoms. Fortunately, a few recent non-pharmacological treatments, including gastric electrical stimulation (GES), have renewed the hope for achieving new management strategies, but the mechanisms underlying their efficacy remain unclear.

Recently, a number of studies have indicated that interstitial cells of Cajal (ICCs) may play an important physiological role in coordinating gastric contractile activity and gastric motility[1,2]. C-kit protein is known to localize on the surface of ICCs and to be activated by interactions between kit receptors and stem cell factor (SCF). SCF/c-kit signaling is important for maintaining the ICC phenotype and ICC proliferation, differentiation and survival[3,4]. Furthermore, some studies have suggested that damage to or the loss of ICCs leads to gastrointestinal motility disorders in diabetic models[5-7] and that decreases in the levels of SCF and insulin-like growth factor 1 (IGF-1) are involved in ICC deficiency[8]. According to the results of recent studies, SCF and IGF-1, which are necessary for the differentiation and maintenance of the ICC phenotype, are derived from smooth muscle cells (SMCs), and IGF-1 increases the expression of SCF[9]. Moreover, the IGF-1 receptor (IGF-1R) appears to be lacking in mature ICCs, and IGF-1 is likely to directly interact with receptors on SMCs to enhance SCF synthesis, resulting in a protective effect in ICCs[8]. Based on a recent study, the levels of serum IGF-1 and its binding protein 3 are altered in type 1 diabetes and that circulating IGF-1 and its binding protein 3 control colonic stem cell function and gastrointestinal complications of diabetes[10].

Generally, GES is classified into three categories according to the length of the pulse and the energy that is applied: the short-pulse GES, the long-pulse GES and the dual-pulse GES. The effects of GES varied with the parameters. In refractory gastroparesis patients who cannot undergo drug therapy due to serious side effects or who are unable to undergo surgical treatment, GES is an alternative option. Our previous study indicated that GES might increase the expression of membrane stem cell factor (M-SCF) in streptozotocin (STZ)-induced diabetic rats[11]. However, the mechanism by which GES affects gastric motility and the involvement of IGF-1 signaling remain unknown. The aim of this study was to: (1) systematically assess the effects of applying GES with different parameters on gastric emptying; (2) ascertain the effects of GES on ICCs in diabetic rats; and (3) determine whether the IGF-1 pathway is involved in the regeneration of ICCs.

MATERIALS AND METHODS

Animal subjects

Adult male Sprague-Dawley rats (250-300 g) obtained from the Experiment Animal Center of Tongji Medical College were used in this study. All appropriate measures were adopted to minimize discomfort or pain to the animals. The rats were housed in standardized laboratory conditions (a 12/12 h light/dark cycle at 22 ℃) and allowed free access to standard solid food and sterile water. The rats were formally enrolled after they were adapted to the laboratory conditions for one week. All experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Tongji Medical College.

Experimental protocols

The animals were randomized into one of six equal groups (6 rats/group): control, diabetic (DM), diabetic and sham GES (DM + SGES), diabetic and GES parameter 1 (5.5 cpm, 100 ms, 4 mA) (DM + GES1), diabetic and GES parameter 2 (5.5 cpm, 300 ms, 4 mA) (DM + GES2), and diabetic and GES parameter 3 (5.5 cpm, 550 ms, 2 mA) (DM + GES3). Diabetes was

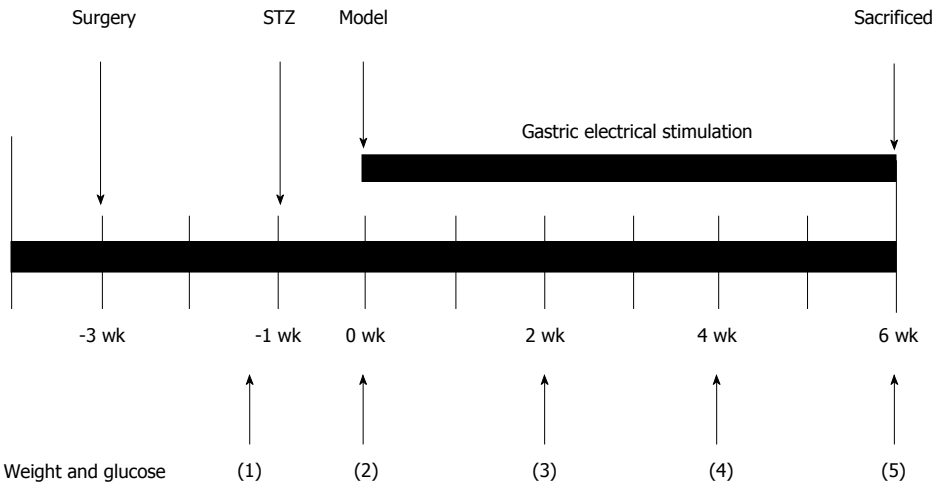

Figure 1 Schematic representation of the gastric electrical stimulation study protocol. After surgery of electrode implantation, the rats had 2 wk to recover from the surgery. After streptozotocin injection, the diabetic rats were randomized into four large groups depending on the parameter of gastric electrical stimulation (GES): DM (diabetic group) + SGES (diabetic with sham GES), DM + GES1 (GES parameter 1), DM + GES2 and DM + GES3 groups for 6 wk. GES was adopted for 30 min/d, 7 d/wk during the whole process of the experiment. The body weights and blood glucose levels were measured before the injection of STZ and during weeks 0, 2, 4, and 6 after the induction of diabetes.

induced using STZ (60 mg/kg, ip, Alexis Biochemical, United States). Rats in the control group were injected with equal volume of diluent. Blood glucose concentrations were measured one week after the injection. The rats were considered diabetic if their blood glucose level increased and was maintained at equal to or more than 16.7 mmol/L. Body weight and blood glucose levels were also tested before the rats were injected and during weeks 0, 2, 4, and 6 after the induction of diabetes. After sequential GES intervention was performed for 6 wk, gastric emptying was measured in all groups. Serum samples were collected for enzyme-linked immunosorbent assay (ELISA), and the rats were sacrificed and the specimens of the antrum were acquired. Each antrum specimen was separated into two pieces. One of these was stored at -80 ℃ for Western blot analysis, and a relatively large piece was fixed in Zamboni's fixative [1.5% saturated picric acid solution and 2% paraformaldehyde in 0.1 M phosphate buffer solution (PBS, pH = 7.4)] for the immunofluorescence study.

Gastric electrical stimulation

Rats in the GES and sham GES groups were anesthetized using pentobarbital sodium (30 mg/kg, ip, Sigma, United States). A midline laparotomy was performed, and stimulating electrodes were pierced the subserosa of the stomach and placed on the serosal surface along the greater curvature. The distal pair of electrodes was about 0.5 cm away from the pylorus and the proximal pair was approximately 1.5 cm away from the distal pair. The electrodes of the proximal pair were placed 0.3 cm apart. The wires were tunneled through the anterior abdominal wall and led out of the skin so they could be connected to the stimulator (G6805-2A; Shanghai Huayi Medical Instrument Factory, China). The abdominal wall was closed using a simple interrupted suture. After the rats recovered from the surgery completely (usually 2 wk), they continuously received GES intervention for 30 min/day for 6 wk (Figure 1). The following respective long-pulse GES frequencies, pulse widths and amplitude parameters were used: GES1, 5.5 cycles/min (cpm), 100 ms and 4 mA; GES2, 5.5 cpm, 300 ms and 4 mA; and GES3, 5.5 cpm, 550 ms and 2 mA. These parameters were shown to be effective and representative in previous experiments[11-13]. Rats in the DM + SGES group were connected only to the stimulator and were not stimulated with an electric current.

Measurement of gastric emptying

The test meals, including carboxymethylcellulose (15 mg/mL) and phenol red (0.5 mg/mL), were continually stirred and maintained at 37 ℃. After the rats were deprived of food overnight, the animals were fed 2 mL of the test meal using a straight gavage needle. After 30 min, the rats were quickly sacrificed using cervical dislocation. The stomach was acquired after it was ligated at the pylorus and cardia, and then it was opened. The gastric contents were placed into a test tube and then washed using distilled water. A NaOH solution (20 mL, 1 mol/L) was placed in each test tube. Absorbance at 560 nm was read using a spectrophotometer (U-2900, Hitachi, Japan) to determine the quantity of remained phenol red.

Gastric emptying was calculated using the following formula: gastric emptying (%) = 100 × (1-X/Y), where X is the absorbance of phenol red measured at 30 min after the test meal, and Y is the absorbance of phenol red in the control rats that were sacrificed immediately after they were fed the test meal[14].

Immunofluorescence studies

The gastric antrum tissues were rapidly fixed in Zamboni's fixative for 24 h at room temperature. They were then dehydrated, embedded in paraffin and cut at a thickness of 5-7 μm. The paraffin sections were deparaffinized and hydrated before antigen retrieval was performed. Endogenous peroxidase was controlled using 3% hydrogen peroxide (H_2O_2) for 30 min. The sections were then microwaved (750 W) for 5 min, and nonspecific reactions were inhibited using normal goat serum for 20 min. The primary antibodies, including IGF-1R (1:200; Santa Cruz Biotechnology, Inc), c-kit (1:150; Santa Cruz Biotechnology, Inc) and α-SMA (1:500; Santa Cruz Biotechnology, Inc), were added dropwise to the paraffin sections which were placed at 4 ℃ overnight. The sections were then rinsed three times using PBS (PH 7.4) and incubated in secondary antibodies for 60 min at 37 ℃, followed by incubation with DAPI (Sigma, United States) for 30 min. The sections were then counterstained and dehydrated. The specimens were observed using a laser scanning confocal microscope (Olympus, Tokyo, Japan).

Western blot analysis

The antrum specimens were homogenized in RIPA buffer (Upstate, United States) containing protease inhibitor (Beyotime, China) and incubated for 30 min on ice. The liquid was centrifuged at 12000 *g* at 4 ℃ for 8 min, and the supernatant containing the total extracted proteins was then collected. BCA reagent (Pierce, Rockford, IL, United States) was used to analyze the protein concentration of each sample. Total proteins were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to PVDF membranes (Millipore, United States). The membranes were immersed in 5% skim milk solution for 1 h and then incubated with primary antibodies against c-kit (1:200, Sigma-Aldrich, United States), SCF (1:200, Abcam, United Kingdom), IGF-1R (1:200, Boster, China) or β-actin (1:1000, Beyotime, China) at 37 ℃ with gentle shaking for 1 h, followed by maintenance at 4 ℃ overnight. After incubation with secondary antibodies for 1 h, the bands on the PVDF membranes were visualized using enhanced chemiluminescence (Amersham Pharmacia, United States). A densitometry analysis was conducted using AlphaView software.

ELISA

For this experiment, approximately 2 mL of blood was obtained from each rat to study differences in serum IGF-1 levels. The concentration of IGF-1 was quantified using rat IGF-1 ELISA kits (RayBiotech, United States). A standard curve was established for IGF-1 by testing the standard with a spectrophotometer at 450 nm, and the concentration of IGF-1 in the serum was then determined by comparing the optical densities of the study samples to those of the standard samples.

Electron microscopy

The gastric antrum was immersed in fixative solution (2.5% glutaraldehyde) for 2 h at 4 ℃. Tissue samples (approximately 1 mm × 5 mm) were separated from the gastric antrum and soaked in 1% OsO_4 for 60-120 min. They were then rinsed with 0.1 mol/L phosphate buffer and dehydrated in ethanol. The tissue samples were immersed in propylene oxide followed by mixtures of Epon Resin and propylene oxide for 2 h, and then embedded in Epon. An ultramicrotome was used to identify the regions of interest in the study and to section them into ultrathin sections (70 nm). The sections were visualized using a transmission electron microscope (Tecnai G212, FEI, The Netherlands).

Statistical analysis

The data are presented as the mean ± SEM. One-way analysis of variance was used to evaluate differences among groups. The least significant difference post hoc test was applied to compare differences between groups. A P value < 0.05 was considered statistically significant. Statistical analyses were performed using SPSS 17.0 software.

RESULTS

Weight and blood glucose level

Baseline weight did not markedly differ between the groups (Figure 2A). The weights of the rats in the DM group were markedly lower at the end of weeks 2, 4 and 6 (P = 0.013, P = 0.005 and P < 0.0001, respectively) than the weights of the controls. The weights of the DM + GES1, DM + GES2 and DM + GES3 groups were significantly higher at the 6th week than the weights in the DM group (P = 0.035, 0.028 and 0.031, respectively).

As shown in Figure 2B, baseline blood glucose levels were not significantly different between the groups. After the induction of diabetes, the blood glucose levels were higher in the DM group than in the control group for the remainder of the experiment (P < 0.0001). After long-pulse GES intervention was applied, blood glucose levels were not markedly different between the DM + GES1, DM + GES2 or DM + GES3 group and the DM group (P = 0.332, 0.281 and 0.416, respectively).

Electron microscopy

In the control group, ICCs showed higher electron-dense cytoplasm than SMCs and were abundant in the endoplasmic reticulum, mitochondria and basal lamina (Figure 3A). In addition, they showed a close connection with SMCs and enteric nerves. However, in the DM group, ICCs were seriously impaired (Figure 3B): swollen endoplasmic reticulum and mitochondria, extensive vacuolization, cytoplasmic depletion and lamellar bodies were frequently observed in their cell bodies. The intercellular spaces were dilated,

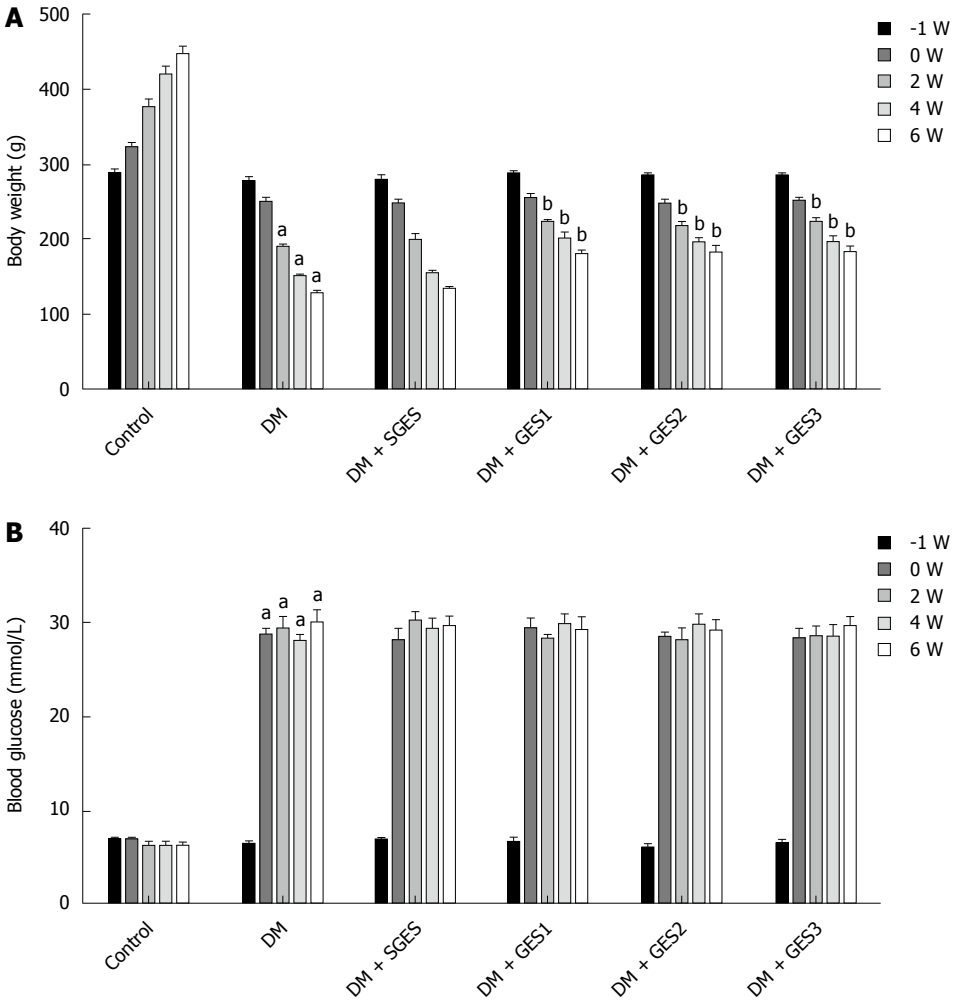

Figure 2 Body weights and blood glucose levels at different time points in different groups. A: The body weights of the diabetic rats were obviously decreased compared with the controls at 6 wk ($P < 0.05$). The weights in all GES groups were increased compared with the DM (diabetic group) ($P < 0.05$). There was no significant difference between the DM and DM + SGES groups ($P > 0.05$); B: Compared with the control group, the blood glucose level of the DM group was significantly increased at weeks 0, 2, 4 and 6 ($P < 0.05$ for all). [a]$P < 0.05$ *vs* control; [b]$P < 0.05$ *vs* DM group.

gap junctions between ICCs and enteric nerves were reduced and exudation of fibrin was occasionally observed. ICCs in the DM + SGES group displayed ultrastructural changes that were similar to those in the ICCs in the DM group (Figure 3C). However, most of the ICCs in the DM + GES1 (Figure 3D), DM + GES2 (Figure 3E) and DM + GES3 (Figure 3F) groups were repaired, and minor damage (the structure of the cytomembrane was relatively complete, organelles were abundant, there were slight cytoplasmic depletion, slightly swollen endoplasmic reticulum and mitochondria and limited vacuolization, there were few denuded ribosomes and a small number of lysosomes, and there was little heterochromatin.) was occasionally observed.

Gastric emptying

Figure 4 shows the effect of GES on gastric emptying. Gastric emptying in the DM group was slower than that in the control group ($P < 0.0001$). SGES did not markedly affect gastric emptying compared to the DM group ($P = 0.573$), but GES1, GES2 and GES3 significantly improved the delayed gastric emptying from 35.89% ± 4.43% to 48.27% ± 2.20%, 61.84% ± 4.87 and 53.34 ± 2.26%, respectively ($P = 0.03$, 0.0028 and 0.0041, respectively). These improvements were significantly different between the DM + GES1 group and the DM + GES2 group ($P = 0.029$), indicating that the effect of GES2 on gastric emptying was stronger than that of GES1.

Western blot analysis of c-kit, SCF and IGF-1R

As shown in Figure 5A and B, the expression of c-kit was evaluated in the antrum. The level of c-kit was markedly lower in the DM group than in the control group ($P < 0.0001$). Conversely, the expression of c-kit was dramatically higher in the DM + GES1, DM + GES2 and DM + GES3 groups than in the DM group ($P = 0.0004$, $P < 0.0001$ and $P = 0.0027$, respectively). The expression of M-SCF (Figure 5C and D) was lower in the DM group than in the control group ($P < 0.0001$). However, GES1, GES2 and GES3 resulted in a higher

Table 1 Serum levels of IGF-1 in each group

	Control	DM	DM + SGES	DM + GES1	DM + GES2	DM + GES3
IGF-1 (ng/mL)	281.22 ± 9.75	31.27 ± 5.61[a]	29.18 ± 5.02	90.88 ± 6.74[c]	121.62 ± 9.97[c,e]	113.97 ± 9.37[c]

Data are given as mean ± SEM. [a]$P < 0.05$, Control *vs* DM group; [c]$P < 0.05$ *vs* DM group; [e]$P < 0.05$, DM + GES2 *vs* DM + GES1 group.

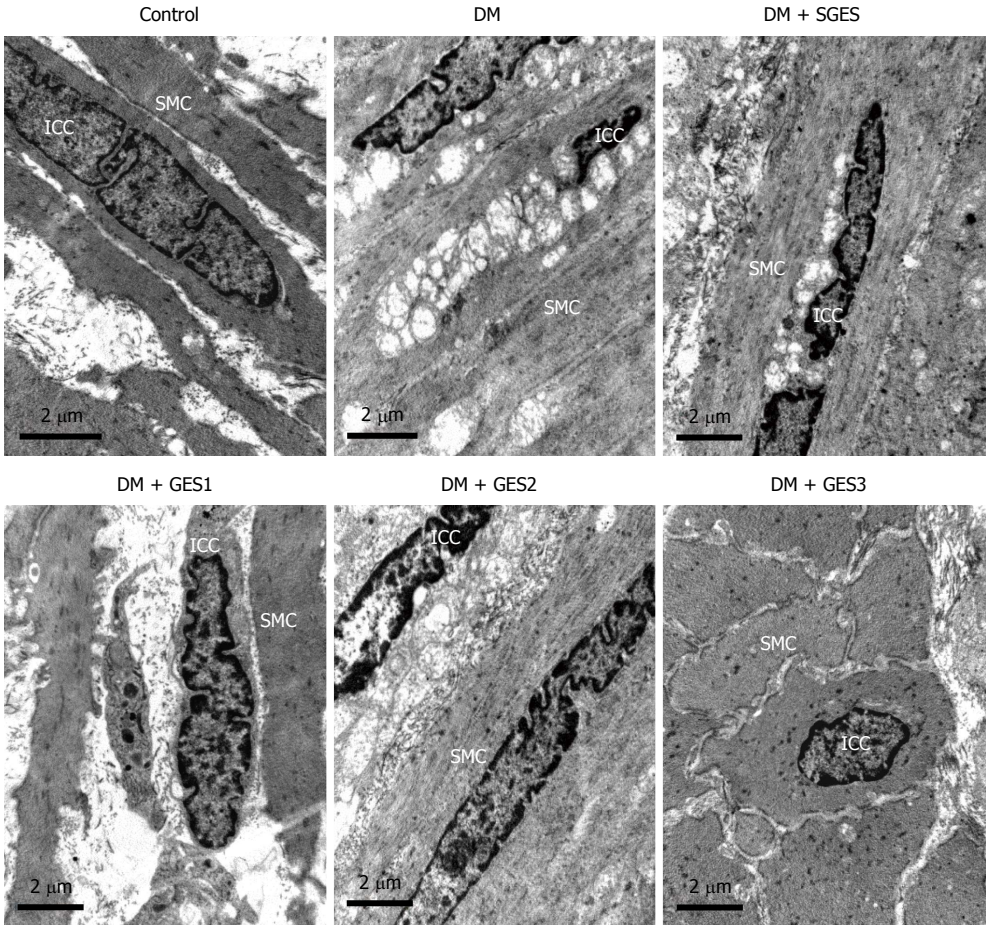

Figure 3 Electron microscopy of interstitial cells of Cajal in each group. In the DM (diabetic) and SGES (diabetic with sham GES) groups, interstitial cells of Cajal (ICCs) were markedly affected compared with the control group, while they appeared to be almost normal in structure or had minor damage in the DM (diabetic group) + GES1 (GES parameter 1), DM + GES2 and DM + GES3 groups. Scale bars = 2 μm.

level of expression of M-SCF than was observed in the DM group ($P = 0.001$, $P < 0.0001$ and $P < 0.0001$, respectively). The effect of GES2 was stronger than that of GES1 ($P = 0.028$).

Compared to the control, the expression of IGF-1R (Figure 5E and F) was markedly lower in the DM group ($P < 0.0001$), whereas the level of IGF-1R in the DM + GES1, DM + GES2 and DM + GES3 groups was significantly higher than the level in the DM group ($P = 0.004$, $P < 0.0001$ and $P = 0.001$, respectively). This effect was stronger in the GES2 group than in the GES1 group ($P = 0.039$).

Serum IGF-1 levels

Table 1 shows the levels of IGF-1 in the serum in each group. The IGF-1 levels in the Control, DM, DM + SGES, DM + GES1, DM + GES2 and DM + GES3 groups were 281.22 ± 9.75, 31.27 ± 5.61, 29.18 ± 5.02, 90.88 ± 6.74, 121.62 ± 9.97 and 113.97 ± 9.37 ng/mL, respectively. The average IGF-1 level in the DM group was lower than that in the control group ($P < 0.0001$). However, the IGF-1 level was higher in the DM + GES1, DM + GES2 and DM + GES3 groups than in the DM group ($P < 0.0001$ for all). The level of IGF-1 was not markedly different between the DM + SGES and the DM groups ($P = 0.794$).

Immunofluorescence studies of c-kit and IGF-1R

As shown in Figure 6, c-kit immunoreactivity revealed the distribution of ICCs in the gastric antrum of each group. There were a number of c-kit$^+$ cells in the control group. However, few ICCs were observed in the DM group. Similar results were found in the DM + SGES group, and long-pulse GES interventions

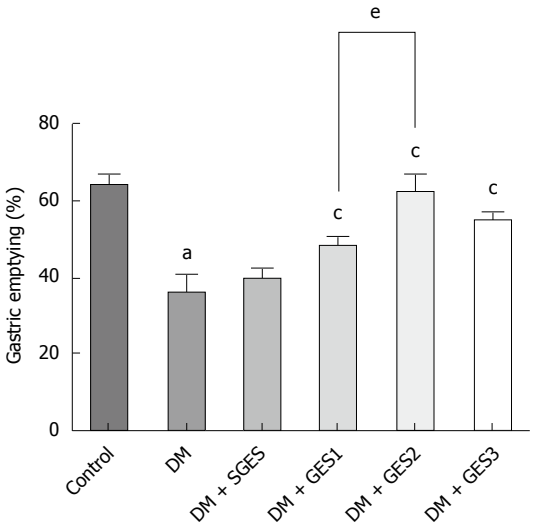

Figure 4 Effects of gastric electrical stimulation on gastric emptying. Compared with the control group, gastric emptying in the DM group was significantly delayed ($P < 0.01$). However, gastric electrical stimulation (GES) significantly improved the delayed gastric emptying ($P < 0.01$ for all). Meanwhile, significance was found between the DM (diabetic group) + GES1 (GES parameter 1) group and DM + GES2 group ($P = 0.0275$). $^{a}P < 0.05$ *vs* control; $^{c}P < 0.05$ *vs* DM group; $^{e}P < 0.05$, DM + GES1 *vs* DM + GES2 group.

markedly increased the expression of c-kit in the DM + GES1, DM + GES2 and DM + GES3 groups.

In Figure 7, α-SMA and IGF-1R immunoreactivity reflects the distribution of smooth muscle cells and IGF-1 receptors, respectively, in the gastric antrum of each group. In the control group, a large number of IGF-1R^{+} cells was observed in the intramuscular layer, whereas few IGF-1R^{+} cells were observed in the DM group. Analogously, few IGF-1R^{+} cells were observed in the DM + SGES group. However, IGF-1R expression was markedly increased in the DM + GES1, DM + GES2 and DM + GES3 groups after treatment with long-pulse GES.

DISCUSSION

In the current study, applying GES using various settings increased the expression of M-SCF and IGF-1/IGF-1R and improved the regeneration of ICCs in diabetic rats, which ameliorated delayed gastric emptying in these rats.

In recent years, GES has been proposed as a therapeutic option for patients suffering from refractory gastroparesis. Some studies have demonstrated that the effects of GES depend on the GES parameters. Short-pulse GES improved dyspeptic symptoms, while long-pulse GES normalized gastric dysrhythmia and regulated gastric slow waves, and dual-pulse GES reduced dyspeptic symptoms and gastric dysrhythmia[15-20]. Because in some instances, long-pulse GES re-establishes normal slow wave activity and improves gastric emptying, it is usually applied to treat gastroparesis. Based on the results of previous studies, a frequency of 5.5 cpm, a pulse width ranging from 100 ms to 300 ms and an amplitude of 4 mA are frequently used and have been shown to be effective in regulating gastric dysrhythmia and gastric slow waves[21,22]. In this study, we selected representative widths of 100 ms (GES1: 5.5 cpm, 100 ms, and 4 mA) and 300 ms (GES2: 5.5 cpm, 300 ms, and 4 mA) for further study. Additionally, GES3 (5.5 cpm, 550 ms, 2 mA) was also demonstrated to be effective in our previous study[11], and that setting was consequently also used in this study. Bellahsène *et al*[23] demonstrated that long-pulse GES (7 cpm, 300 ms, 4 mA) accelerated gastric emptying in a canine model of gastroparesis. Moreover, McCallum *et al*[24] discovered that long-pulse GES (at a frequency that was 10% higher than the intrinsic slow-wave frequency, 300 ms, 4 mA) accelerated gastric emptying in gastroparesis patients. However, Xing *et al*[20] found that long-pulse GES (6 cpm, 375 ms, 4 mA) did not significantly influence gastric emptying in a canine model. In the present study, delayed gastric emptying was accelerated after GES intervention, especially in the GES2 group (5.5 cpm, 300 ms, 4 mA), which supports the findings of most studies in this field. Our results showed that long-pulse GES improved delayed gastric emptying in diabetic rats, and the effect of GES2 (5.5 cpm, 300 ms, 4 mA) was more pronounced than those of GES1 and GES3. Thus, the GES2 protocol should potentially be applied in the clinic, but further studies are needed to explore this issue.

The mechanisms by which long-pulse GES affects gastric emptying remain unclear. ICCs have been shown to play an important role in the regulation of gastric peristalsis, which significantly affects gastric emptying[1,25]. However, few studies have examined the involvement of ICCs in the effect of long-pulse GES on gastric emptying. We conducted related experiments in which we examined the effect of GES on ICCs. The results revealed that long-pulse GES (5.5 cpm, 550 ms, 2 mA) resulted in ICC remodeling in diabetic rats. Furthermore, our previous study showed that both low- and high-frequency electroacupuncture at ST-36 increased the number of ICCs[14]. In the present study, GES intervention also markedly recovered the ultrastructure of ICCs to a minor injury or normal state[26] and increased the number of ICCs in the antrum in diabetic rats, indicating that long-pulse GES might induce ICC remodeling and further contribute to improved gastric emptying.

The c-kit/SCF pathway is one of the most important regulators of ICCs. As the ligand of c-kit, SCF exists in both a soluble form (S-SCF) and a transmembrane form (M-SCF). M-SCF may play a more important role than S-SCF during the differentiation, survival and maintenance of the ICC phenotype[27]. Thus, we focused on M-SCF in our evaluation of c-kit/SCF signaling and our goal of identifying the mechanisms by which GES results in ICC remodeling. Horváth *et al*[8] linked reduced SCF to an ICC deficiency in diabetic gastroparesis and found that the level of M-SCF was

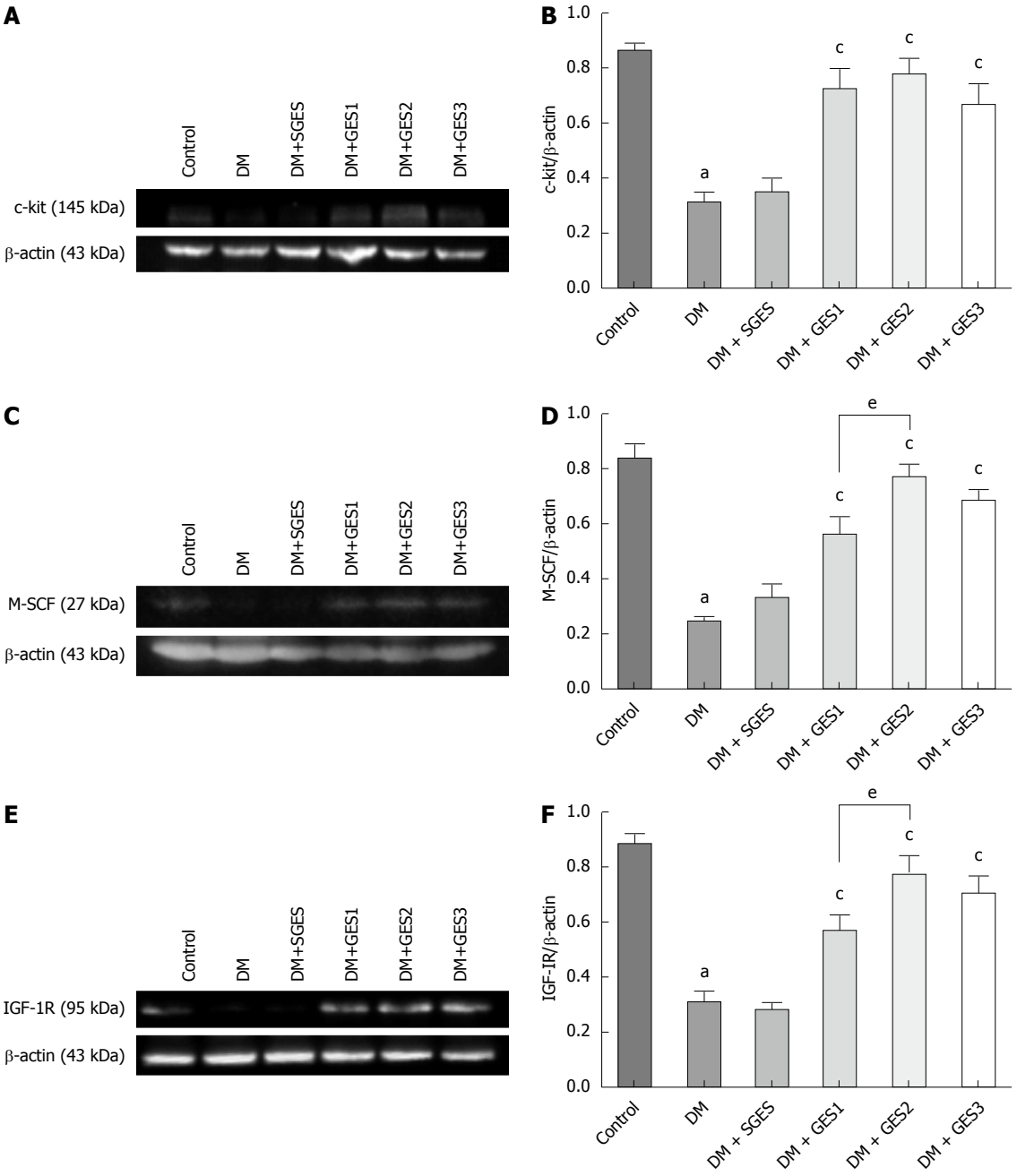

Figure 5 Expression levels of related proteins quantified by Western blot. A, B: The expression of c-kit in different groups; C, D: The expression of M-SCF in different groups; E, F: The expression of IGF-1R in different groups. $^{a}P < 0.05$ *vs* control; $^{c}P < 0.05$ *vs* DM (diabetic group) group; $^{e}P < 0.05$, DM + GES1 (GES parameter 1) *vs* DM + GES2 group. GES: Gastric electrical stimulation.

decreased in the gastric antrum in diabetic mice. Our results also indicate that the expression of M-SCF is markedly decreased in diabetic rats. However, the effects of GES on the expression of M-SCF have rarely been investigated. In a previous study, we showed that both low- and high-frequency electroacupuncture at ST-36 increased the expression of M-SCF in diabetic rats[14]. In the present study, we found that GES treatment also increased the expression of M-SCF, and this increase was stronger when GES2 (5.5 cpm, 300 ms, 4 mA) was applied. Therefore, M-SCF is likely to be involved in ICC remodeling as a result of long-pulse GES.

IGF-1 signaling is one of the main factors that regulate the expression of M-SCF. IGF-1R, which is necessary for the differentiation and maintenance of ICC phenotype, is located on smooth muscle cells but not on ICCs[8,28]. Horváth *et al*[29] showed the first *in vitro* evidence that IGF-1 signaling was responsible for maintaining the ICC network. In their subsequent study, they suggested that endogenous IGF-1 expression was decreased in the diabetic group and that adding IGF-1 to the medium improved the expression of M-SCF[9]. The results of the present study show that the expression of IGF-1R in gastric antral SMCs was markedly decreased in the DM group,

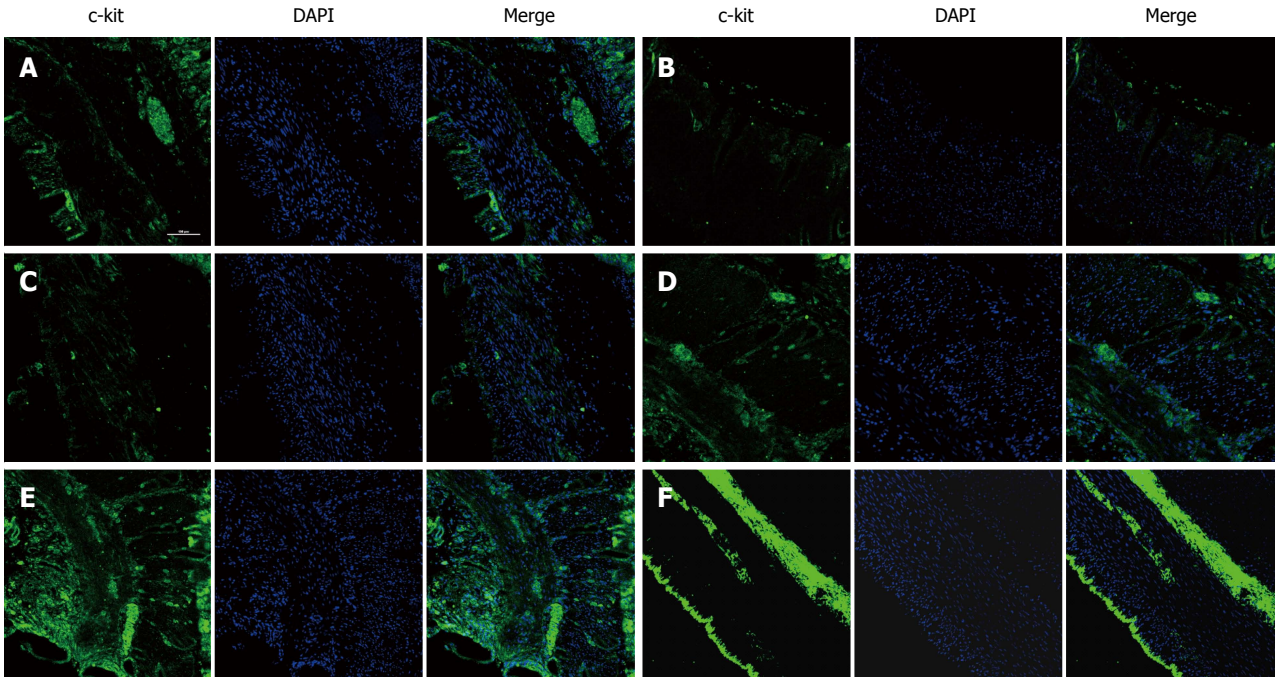

Figure 6 Confocal micrographs of interstitial cells of Cajal. Interstitial cells of Cajal (ICCs) were labelled with c-kit (green) and DAPI (blue). ICCs were abundant in the control group (A). Few ICCs were observed in the DM group (B). Similar changes in ICCs were found in the SGES (diabetic with sham GES) group (C). However, numerous ICCs were observed in the DM (diabetic group) + GES1 (GES parameter 1) group (D), DM + GES2 group (E) and DM + GES3 group (F). Scale bars = 100 μm. GES: Gastric electrical stimulation.

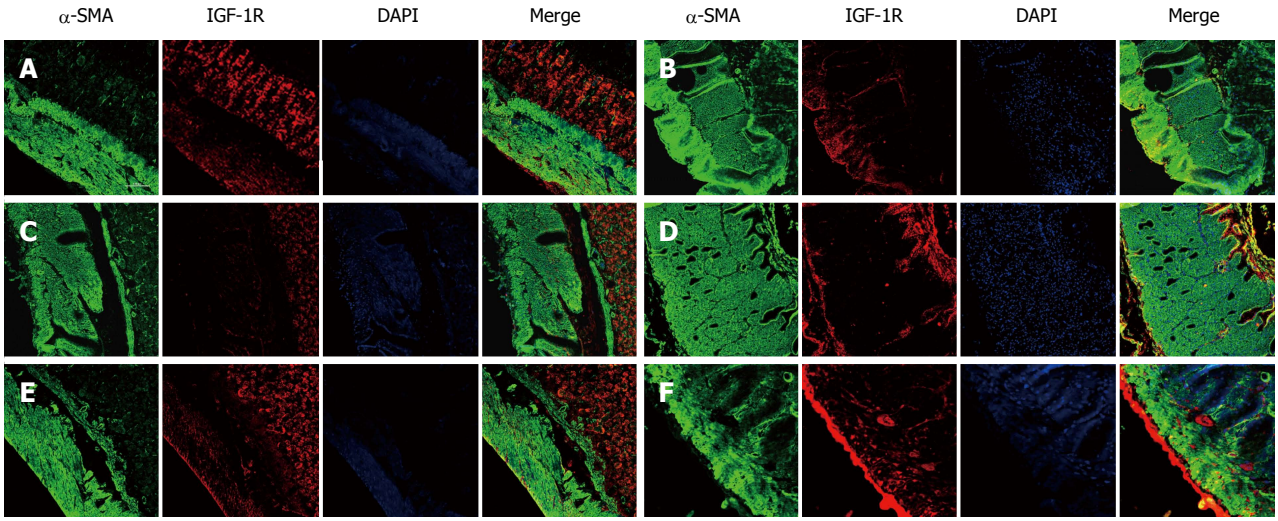

Figure 7 Confocal micrographs showing the distribution of insulin-like growth factor 1 receptor. The smooth muscle cells were labelled with α-SMA (green), IGF-1R (red) and DAPI (blue). IGF-1R expression was high in the control group (A). Weak IGF-1R signals were found in the DM group (B), and similar changes appeared in the SGES group (diabetic with sham GES) (C). Strong IGF-IR signals were captured in the DM (diabetic group) + GES1 (GES parameter 1) group (D), DM + GES2 group (E) and DM + GES3 group (F). Scale bars = 100 μm. DM: and GES parameter 1. GES: Gastric electrical stimulation; IGF: Insulin-like growth factor.

which supports the findings of previous studies[8,9]. Moreover, serum IGF-1 levels were also decreased in the DM group. However, few studies have examined the effects of GES on IGF-1 and IGF-1R levels or the involvement of IGF-1 signaling in the induction of ICC remodeling by GES. In our study, the levels of both IGF-1 and IGF-1R were significantly increased following GES treatment, which mirrored the changes we observed in M-SCF. Additionally, GES2 (5.5 cpm, 300 ms, 4 mA) was more effective in improving the expression of IGF-1 and IGF-1R. These results indicate that improvements in IGF-1 signaling might be involved in the up-regulation of M-SCF expression and that it might also contribute to ICC remodeling in response to long-pulse GES in diabetic rats.

Xing *et al*[30] reported that the total area under the curve for blood glucose in healthy dogs was markedly decreased by long-pulse GES (10 cpm, 300 ms, 8 mA). In the present study, GES did not significantly affect blood glucose levels. We speculate that the

difference between these effects might be associated with differences in the parameters, animal models and physical states that were used, and that the effect of GES on the blood glucose levels in diabetic models might therefore require further study. Yan *et al*[31] reported that pulse-train GES (0.3 ms, 3 mA, 20 Hz for 2 s on and 3 s off) reduced body weight in obese rats. Our results show that long-pulse GES treatment markedly increased body weight in diabetic rats. Therefore, the effects of GES on body weight might depend on the parameters and physical states that are used. Broadly, our results suggest that GES increased the body weight but did not significantly affect blood glucose levels in STZ-induced diabetic rats.

In summary, our results reveal that the expression levels of M-SCF, IGF-1 and IGF-1R are significantly lower and that the number of ICCs is markedly decreased in the gastric antrum of diabetic rats. These characteristics likely contribute to delayed gastric emptying. Following long-pulse GES intervention, ICCs remodeled and gastric emptying was improved. The IGF-1 signaling pathway might participate in this process by enhancing the expression of M-SCF on SMCs and then protecting ICCs. Furthermore, the effects of GES2 (5.5 cpm, 300 ms, 4 mA) on the expression of M-SCF and IGF-1/IGF-1R appeared to be stronger than those of GES1 and GES2. Long-pulse GES involves low-frequency/high-energy stimulation; its frequency is slightly higher than the intrinsic slow wave and requires a high amount of energy. This stimulus could directly activate ICCs and/or SMCs without involving cholinergic nerves[21], and the magnitude of energy is probably responsible for the effects of long-pulse GES. In our study, GES2 (5.5 cpm, 300 ms, 4 mA) afforded higher energy than did GES1 (5.5 cpm, 100 ms, 4 mA) and GES3 (5.5 cpm, 550 ms, 2 mA), and this might partly explain why GES2 had a more significant effect on gastric emptying and the IGF-1 signaling pathway. Thus, this parameter should be used in clinical applications involving long-pulse GES. However, further studies into the mechanisms underlying these processes are needed to ensure the safety and clinical efficacy of using long-pulse GES as a treatment for refractory functional gastric disorders.

COMMENTS

Background

Gastric electrical stimulation (GES) regulates gastric motility and promotes the renovation of interstitial cells of Cajal (ICCs). However, the mechanisms underlying this effect remain unclear. Previous studies demonstrated that ICCs are damaged and the insulin-like growth factor 1 (IGF-1) pathway is down-regulated in diabetic models. In this study, the authors report that applying long-pulse GES using three different parameters promoted the regeneration of ICCs and that the IGF-1 signaling pathway is likely to be involved in this process.

Research frontiers

Experiments have shown that ICCs are damaged in diabetic models. The c-kit/SCF pathway is one of the most important regulators of ICCs. M-stem cell factor (SCF) may play a more important role than S-SCF in the differentiation, survival and maintenance of the ICC phenotype. IGF-1 signaling is one of the main factors that regulate the expression of M-SCF.

Innovations and breakthroughs

This study provides evidence showing that long-pulse GES protects the ICCs in diabetic rats and that the IGF-1 signaling pathway is involved in this effect. The effects of one GES parameter (5.5 cpm, 300 ms, 4 mA) were stronger.

Applications

One GES parameter (5.5 cpm, 300 ms, 4 mA) showed greater potential for being used in clinical application involving long-pulse GES, and the IGF-1 signaling pathway may be a possible therapeutic target in gastroparesis.

Terminology

ICCs, as the gastrointestinal pace-making cells, play an important physiological role in coordinating gastric contractile activity and gastric motility. Long-pulse GES restores injured ICCs. SCF and IGF-1 are regulatory proteins that protect ICCs.

Peer-review

An interesting paper! The paper by Li and colleagues describes the effect of different timing pulse GES (gastric electrical stimulation) on gastric emptying in a rat diabetic model. In particular, the authors demonstrate that long-term pulsing GES protects ICCs located in the gastric antrum and this is associated with an improvement of gastric emptying delay during diabetes. Interestingly, the authors also suggest that the IGF-1 signaling pathway may be involved.

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ORIGINAL ARTICLE

Basic Study

MicroRNA-548a-5p promotes proliferation and inhibits apoptosis in hepatocellular carcinoma cells by targeting Tg737

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Author contributions: Zhao G, Wang T and Huang QK performed the majority of experiments; Zhao G, Pu M, Sun W and Zhang ZC provided technical supports and were also involved in editing the manuscript; Zhao G and Tao KS collected all the human materials and provided financial support for this work; Ling R and Tao KS designed the study and wrote the manuscript.

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Institutional review board statement: All procedures were performed in accordance with a protocol approved by Institutional Medical Ethics Committee (No. XJYYLL-2012475).

Institutional animal care and use committee statement: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Fourth Military Medical University (No. 20120211121). All the patients provided written informed consent for the use of specimens included in the study.

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Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at taoks2015@sina.com. Participants gave informed consent for data sharing. No additional data are available.

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Abstract

AIM: To investigate whether Tg737 is regulated by microRNA-548a-5p (miR-548a-5p), and correlates with hepatocellular carcinoma (HCC) cell proliferation and apoptosis.

METHODS: Assays of loss of function of Tg737 were performed by the colony formation assay, CCK assay and cell cycle assay in HCC cell lines. The interaction between miR-548a-5p and its downstream target, Tg737, was evaluated by a dual-luciferase reporter assay and quantitative real-time polymerase chain reaction. Tg737 was then up-regulated in HCC cells to evaluate its effect on miR-548a-5p regulation. HepG2

cells stably overexpressing miR-548a-5p or miR-control were also subcutaneously inoculated into nude mice to evaluate the effect of miR-548a-5p up-regulation on *in vivo* tumor growth. As the final step, the effect of miR-548a-5p on the apoptosis induced by cisplatin was evaluated by flow cytometry.

RESULTS: Down-regulation of Tg737, which is a target gene of miR-548a-5p, accelerated HCC cell proliferation, and miR-548a-5p promoted HCC cell proliferation *in vitro* and *in vivo*. Like the down-regulation of Tg737, overexpression of miR-548a-5p in HCC cell lines promoted cell proliferation, increased colony forming ability and hampered cell apoptosis. In addition, miR-548a-5p overexpression increased HCC cell growth *in vivo*. MiR-548a-5p down-regulated Tg737 expression through direct contact with its 3′ untranslated region (UTR), and miR-548a-5p expression was negatively correlated with Tg737 levels in HCC specimens. Restoring Tg737 (without the 3′UTR) significantly hampered miR-548a-5p induced cell proliferation, and rescued the miR-548a-5p induced cell proliferation inhibition and apoptosis induced by cisplatin.

CONCLUSION: MiR-548a-5p negatively regulates the tumor inhibitor gene Tg737 and promotes tumorigenesis *in vitro* and *in vivo*, indicating its potential as a novel therapeutic target for HCC.

Key words: microRNA-548a-5p; Tg737; Proliferation; Apoptosis; Hepatocellular carcinoma cells

Core tip: Tg737 gene functions as a tumor suppresser in hepatocellular carcinoma (HCC). However, studies based on regulation of Tg737 were rare. MiR-548a-5p is a novel miRNA which negatively regulates Tg737 and promotes tumorigenesis *in vitro* and *in vivo*, indicating its potential as a novel therapeutic target for HCC.

Zhao G, Wang T, Huang QK, Pu M, Sun W, Zhang ZC, Ling R, Tao KS. MicroRNA-548a-5p promotes proliferation and inhibits apoptosis in hepatocellular carcinoma cells by targeting Tg737. *World J Gastroenterol* 2016; 22(23): 5364-5373 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i23/5364.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i23.5364

INTRODUCTION

Primary hepatocellular carcinoma (HCC) is currently one of the most common and lethal malignancies, and the leading cause of death among patients with cirrhosis[1]. Despite advances in surgical therapies, the reason for frequent recurrence remains mostly obscure. Several studies showed that some genes may play a major role in cell proliferation and migration in HCC cells[2]. However, little information is available about the regulation of these genes.

The Tg737 gene, first found in algae[3], is now identified as a tumor suppressor gene in multiple cancers, including cancers of the liver, kidney and pancreas[4-5]. In liver tissues from HCC patients, a 59% down-regulation of the Tg737 was observed[6]. This gene may participate in alterations in a series of human or rodent liver tumors and tumorigenic cell lines[7]. In the previous investigation, we found that Tg737 contributed to hypoxia-induced invasion and migration in HepG2 and MHCC97H cells[8], and that Tg737 inhibition resulted in malignant transformation in fetal liver stem/progenitor cells by promoting cell-cycle progression and differentiation arrest[9]. However, the regulatory factor for Tg737 is not yet clear.

In recent years, microRNAs (miRNAs) emerged as a group of important endogenous modulators of gene function at the posttranscriptional level. It has been revealed that many miRNAs are underscored by the promotion of tumorigenesis and cancer progression[10-11]. More importantly, in HCC, several miRNAs have been demonstrated to contribute to the tumor regulation, including, but not limited to, migration, invasion and proliferation[12-13]. Multiple miRNAs have been identified as down-regulated tumor-suppressing genes involved in cellular processes, including miR-34a[14], miR-122[15], miR-199a[16] and miR-200[17]. Contrarily, miR-21[18], miR-148a[19] and miR-221[20], as oncogenic miRNAs, showed up-regulated expression which potentially targeted many tumor-suppressive genes. These results suggest the involvement of miRNAs in HCC.

MiR-548 is a big, poorly conserved primate-specific miRNA family. There are 68 members of the hsa-miR-548 family recorded in the miRBase database[21]. The products of miR-548c-5p and miR-548c-3p show discrepant evolutionary patterns, which brings about great genetic distances between pre-miRNAs to some extent and might contribute to dynamic expression profiles and regulatory network[22]. MiR-548c-3p was identified as a source of functional biomarkers for the primary prostate cancer progression[23]. It also significantly increased in *Helicobacter pylori*-negative cancer tissues[24]. In addition, our previous studies found the high expression of miR-548c-3p and miR-548c-5p in side population cells from HCC[25]. However, like the homologous gene, the exact effect of miR-548a-5p on HCC is still unknown. The direct aim of this study was to investigate whether Tg737 is regulated by miR-548a-5p, and correlates with HCC cell proliferation and apoptosis.

MATERIALS AND METHODS

Cell lines and culture

HCC cell lines HepG2 and MHCC97-H were maintained in DMEM medium (Invitrogen, United States) sup-

plemented with 10% fetal bovine serum (FBS, Invitrogen, United States), 100 IU/mL penicillin, and 100 μg/mL streptomycin. Cells were cultured in a humidified cellular incubator (Thermo Fisher, United States) supplemented with 5% CO_2 at 37 ℃.

RNA isolation, reverse transcription and quantitative real-time polymerase chain reaction

Total RNA was extracted using RNAiso Plus (Takara, Japan) according to the manufacturer's instructions. Reverse transcription was carried out using the PrimeScript RT Reagent Kit Perfect Real Time (Takara, Dalian, China) or the miScript II RT Kit (Qiagen, Germany). Quantitative polymerase chain reaction (qPCR) was used to detect the miRNA expression levels. The SYBR Green dye (Takara, Dalian, China) was used according to the manufacturer's protocol. The following primers were used for analysis: TG737: forward primer, 5'-GTGCCAGTAGTAAAGGTG-3' and reverse primer, 5'-GGTCGTTCTATTTGAGGG-3'; β-actin: forward primer, 5'-TCACCAACTGGGACGACA-3' and reverse primer, 5'-ACAGCACCGCCTGGATAG-3'; miR-548a-5p: forward primer, 5'-AAAAGTAATTGCGAGTTTACC-3' and reverse primer, Universal Primer (Qiagen, Germany). The CFX96 real-time PCR detection system (Bio-Rad, United States) was used to perform real-time PCR and the $2^{-\Delta\Delta CT}$ method was used to determine the relative gene expression. Mature miRNA was normalized to U6-snRNA.

Plasmid construction

MiR-548a-5p was obtained from Gene Pharma (Shanghai, China). The miR-548a-5p and 150 bp of flanking sequence was amplified with forward primer, 5'-CAGCTGGGTGCTCAGCCAGG-3', reverse primer, 5'-GGCAACTTAATGTTTCTTGC-3'. A PCR fragment was inserted into the pENTRTM3C vector (Invitrogen, United States) using EcoR I and Xho I sites. The pLenti6.3-miR-548a-5p vector was constructed using Gateway LR Clonase II Enzyme Mix (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. The constructs were sequenced for verification. Overexpression and RNAi vectors were determined by Gene Pharma (Shanghai, China). The target sequence of sh-Tg737 was 5-'GTTACATACTTGAGACAAA-3'. Tg737 and Tg737-3'UTR overexpression vectors were also designed.

Western blot

Proteins were extracted from cells with a RIPA buffer purchased from Beyotime with 1 mmol/L PMSF and a cocktail of protease inhibitors. The proper amount of loading buffer was added into cell lysis, followed by a BCA protein determination. SDS-PAGE was performed according to Genshare's instructions (Genshare, Xi'an, China). The results were analyzed using Image J 5.0 software. Anti-Tg737 antibody (13967-1-AP) and anti-β-actin antibody (14395-1-AP) were purchased from Proteintech (Proteintech, Wuhan, China). Goat anti-rabbit IgG HL (HRP) (ab6721) was supplied by Abcam (United States).

Colony formation assay

Two hundred cells were seeded into a 6 cm dish and cultured in a complete medium for 10 d. Cells were fixed in methanol and stained with crystal violet, washed with PBS three times before fixing and staining. Colonies were washed with ddH_2O after 30-min staining. The number and area of the colonies were calculated with Image J 5.0 software.

Cell viability assay

Cells were seeded in 96-well plates (2.0×10^3 cells/well). The cells were incubated for 0, 1, 2, 3 and 4 d using three replicates. Cell Counting Kit-8 (CCK-8) (7Sea Pharmatech, China) was used for cell proliferation analysis according to the manufacturer's protocol. The Bio-Rad iMARK™ microplate reader was used to detect the optical density at 450 nm.

Cell cycle and apoptosis assay

Cells were seeded in 6-well plates with 2.0×10^5 cells in each well. After 48 h, flow cytometry was performed to detect cell cycle analysis and the percentage of dying cells. The annexin V-FITC/PI apoptosis detection kit (Biovision, Palo Alto, CA, United States) was used according to the manufacturer's protocol. Flow cytometry was performed with the Epics XL-MCL (BECKMAN coulter, United States). The results were analyzed using ModFit LT V3.1 (BECKMAN coulter).

Luciferase reporter assays

HepG2 and MHCC97H cells were seeded in 48 well plates and allowed to be adherent for 24 h. The cells were then co-transfected with a mixture of 100 ng pGL3-Tg737-3'UTR, 20 μmol miR-548a-5p mimic or antisense-miR-548a-5p, and 5 ng pRL-TK using Lipofectamine 2000 reagent and performed in three independent experiments. Firefly and Renilla luciferase activities were detected using the Dual-Glo luciferase assay system (Promega, United States) following the manufacturer's instructions.

Tumor xenograft

HepG2 cells (2×10^6) stably overexpressing miR-548a-5p or miR-control were injected subcutaneously into the flank site of male Balb/c nude mice (Fourth Military Medical University, Xi'an, China). Tumor volume was measured with calipers every 5 d[26]. On day 30, mice were killed by cervical dislocation. All animal procedures were performed in accordance with a protocol approved by the Fourth Military Medical University Animal Care and Usage Committee.

Statistical analysis

Sample capacity for each experiment was adjusted according to the variance obtained. All experiments were performed in triplicate. In graphs, all data are

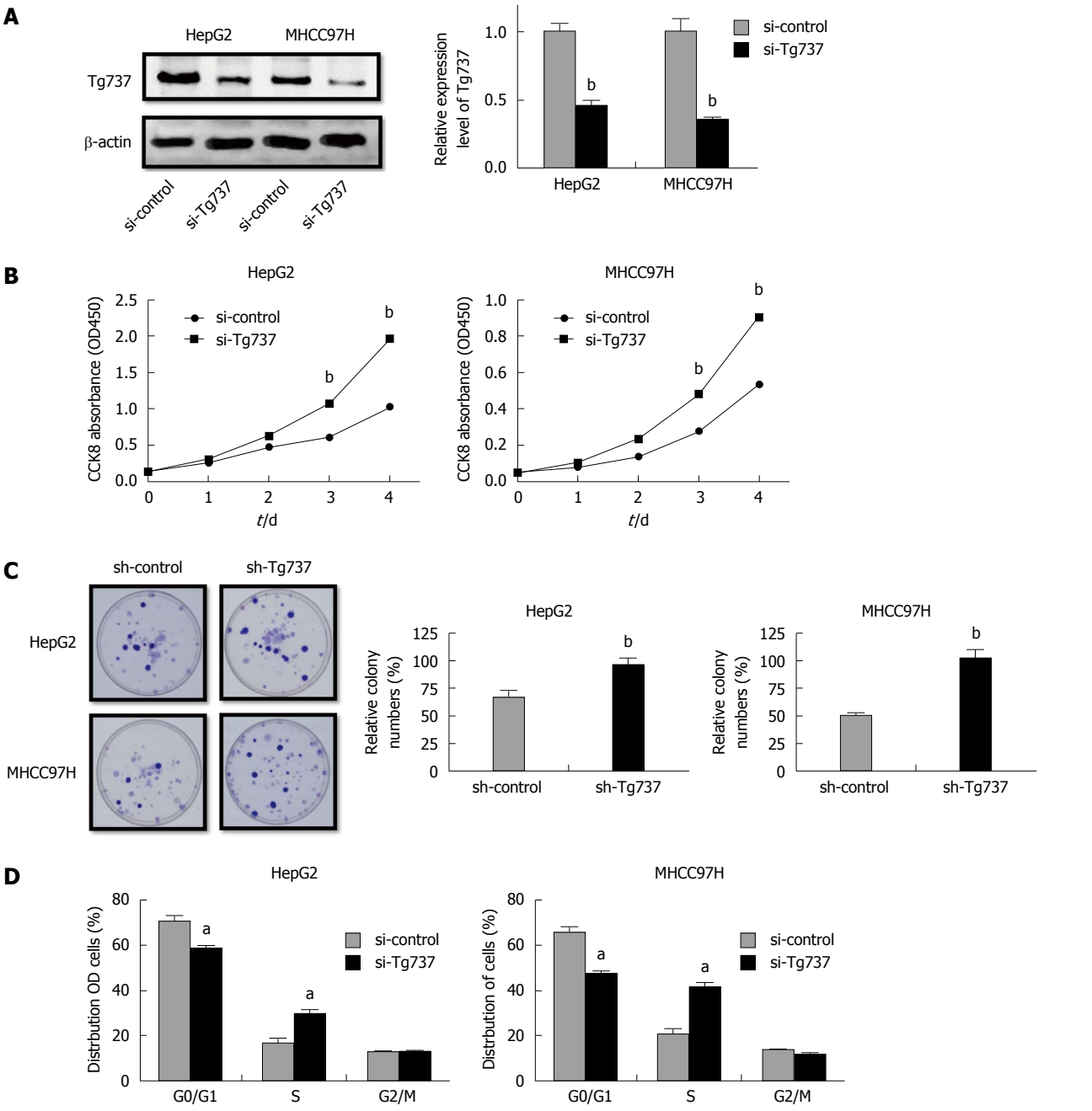

Figure 1 Down-regulation of Tg737 accelerates hepatocellular carcinoma cell proliferation. A: Western blot analysis of Tg737 expression in HepG2 and MHCC97H cells transfected with si-Tg737 or negative control (si-control); B: Impact of Tg737 down-regulation on HCC cell proliferation detected by CCK-8 assay after si-Tg737 and si-control transfection in HepG2 and MHCC97H cell lines; C: Impact of Tg737 down-regulation on colony forming ability of HepG2 and MHCC97H cell lines. The relative percentage of colony numbers from the si-Tg737 group is given as 100%; D: Cell cycle assay of HepG2 and MHCC97H cells transfected with si-Tg737 and si-control. The charts were illustrated by the percentage of cells in the G0/G1, S, and G2/M phase of cell cycle, respectively. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control.

presented as mean ± SD and were evaluated by the Student's *t*-test. Statistical analyses were carried out using SPSS 16.0 software. Differences were considered significant at $P < 0.05$.

RESULTS

Down-regulation of Tg737 promotes HCC cell proliferation in vitro

To illuminate the role of Tg737 in HCC cell proliferation, HCC cell lines HepG2 and MHCC97H were transfected with a si-Tg737 sequence or negative control (Figure 1A). Down-regulation of Tg737 significantly promoted the proliferation of HepG2 and MHCC97H cells and enhanced colony forming capability (Figure 1B and C). The distribution of HepG2 and MHCC97H cell cycles showed that the percentage of cells in G0/G1 phase significantly decreased in Tg737 down-regulated cells compared with their counterparts, while the cells in S phase increased sharply (Figure 1D). In all cases, down-regulation of Tg737 promoted cell proliferation and inhibited G0/G1 phase arrest in HCC cells.

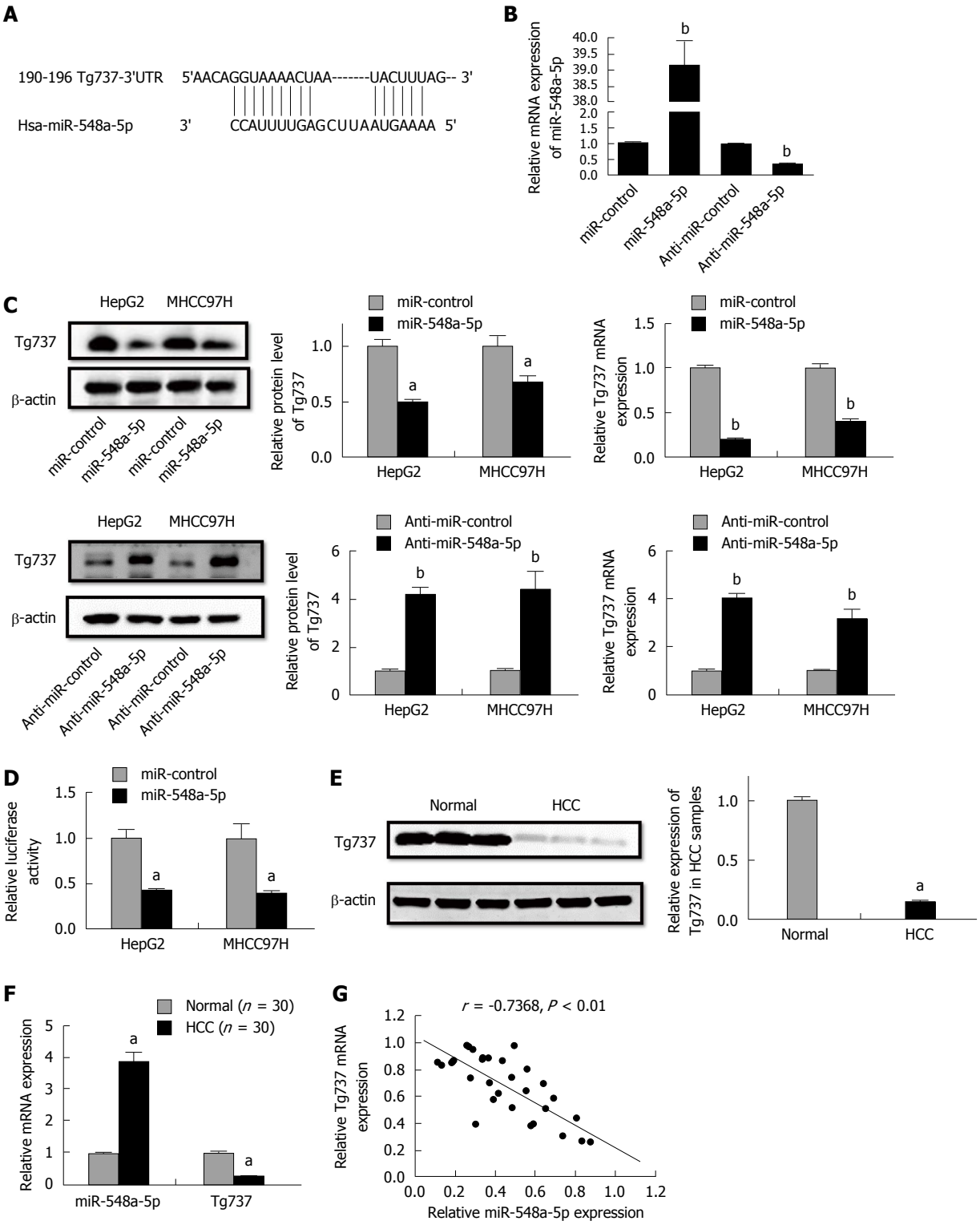

Figure 2 Tg737 is a target gene of miR-548a-5p. A: MiR-548a-5p and its deductive binding domain in the 3'UTR of Tg737; B: Relative expression of miR-548a-5p; C: qRT-PCR and Western blot analysis demonstrated that miR-548a-5p overexpression significantly decreased Tg737 mRNA expression and miR-548a-5p knockdown significantly increased Tg737 mRNA expression in HepG2 and MHCC97H cells; D: Luciferase reporter assay of the connection between the 3'UTR of Tg737 and miR-548a-5p in HepG2 and MHCC97H cells; E: Western blot analysis demonstrated higher miR-548a-5p and lower Tg737 expression in human HCC specimens compared to normal liver tissue; F: qRT-PCR demonstrated higher miR-548a-5p and lower Tg737 expression in human HCC specimens compared to normal liver tissue; G: Spearman's correlation assay showed that miR-548a-5p was negatively correlated with Tg737 mRNA expression in HCC specimens. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control.

MiR-548a-5p down-regulates Tg737 by interacting with its 3'UTR

A bio-informatics assay with TargetScan demonstrated that Tg737 is a potential target of miR-548a-5p (Figure 2A). To illuminate whether miR-548a-5p acts on Tg737, we transfected miR-548a-5p as well as anti-miR-548a-5p to HCC cells. Relative miR-548a-5p levels are shown in Figure 2B. MiR-548a-5p overexpression decreased

mRNA and protein levels of Tg737, while miR-548a-5p inhibition increased mRNA and protein levels of Tg737 in HepG2 and MHCC97H cells (Figure 2C). To confirm whether Tg737 acts as a molecular target regulated by miR-548a-5p, we constructed luciferase reporter vectors containing Tg737-3'UTR. The reporter vectors were co-transfected into HepG2 and MHCC97H cells. Up-regulation of miR-548a-5p expression significantly decreased the luciferase activity of Tg737 containing 3' UTR (Figure 2D). Further, to illuminate whether miR-548a-5p inhibits Tg737 in patients diagnosed with HCC, we detected the expression of miR-548a-5p and Tg737 in 30 HCC specimens as well as 30 normal ones. Compared with normal liver tissues, the HCC specimens showed higher miR-548a-5p and lower Tg737 expression (Figure 2E and F). In addition, a statistically significant correlation was revealed by Spearman's correlation analysis between mRNA levels of miR-548a-5p and Tg737 (r = -0.7368, P < 0.01; Figure 2G). Together, these data suggest that Tg737 is a target of miR-548a-5p in HCC.

MiR-548a-5p promotes HCC cell proliferation in vitro and in vivo

Continuing our analysis, we detected the impact of miR-548a-5p on HCC cell proliferation. Similar to the effect of Tg737 knockdown, miR-548a-5p overexpression in HepG2 and MHCC97H cells significantly accelerated cell proliferation, promoted colony forming capacity, and inhibited cell arrest in G0/G1 phase (Figure 3A-C). To investigate the relationship between miR-548a-5p and its tumor promoting characteristics *in vivo*, HepG2 cells exhibiting sustained expression of miR-548a-5p or control cells were injected into the subcutaneous skin layer of nude mice. Tumor size was detected every 5 d from the injection. The mice were killed 30 d after implantation. As shown in Figure 3D, tumors in those specimens with miR-548a-5p overexpression were larger significantly than those in the control group. The average size and weight of miR-548a-5p overexpressing tumors were increased significantly (Figure 3D and E). Collectively, miR-548a-5p may have a vigorous ability to enhance HCC cell proliferation *in vivo* and *in vitro*.

Restoration of Tg737 inhibits miR-548a-5p-mediated HCC cell proliferation inhibition and protects against cisplatin induced apoptosis

We further traced whether the restoration of Tg737 could arrest the HCC cell proliferation induced by miR-548a-5p overexpression in HepG2 and MHCC97H cells. A Western blot technique was performed 48 h after transfection of control, Tg737 and Tg737-3' UTR overexpression vectors into miR-548a-5p overexpressing HepG2 and MHCC97H cells (Figure 4A). Tg737 up-regulation significantly decreased the capacity of cell proliferation and colony formation (Figure 4B). Up-regulation of Tg737 rescued miR-548a-5p induced inhibition in cell cycle arrest (Figure 4B-D). However, overexpression of Tg737-3'UTR in miR-548a-5p overexpressing HepG2 and MHCC97H cells had no significance (Figure 4B-D). These results again illustrate that Tg737 is a functional target of miR-548a-5p. We evaluated the impact of miR-548a-5p on apoptosis by flow cytometry. Cell apoptosis increased in anti-miR-548a-5p transfected HCC cells (Figure 4E). Meanwhile, the cell apoptosis ratio decreased in miR-548a-5p transfected HCC cells (Figure 4F).

DISCUSSION

To our knowledge, this is the first report describing the effects of miR-548a-5p on HCC cell proliferation and apoptosis and the relationship with the Tg737 protein. In this study, we showed that Tg737 was the key regulator of the proliferation of human HCC cell lines. Overexpression of miR-548a-5p observably increased oncogenesis in nude mice. Moreover, when the expression of Tg737 was inhibited through interaction with its 3'UTR, HCC cells grew quickly when induced by miR-548a-5p. These findings suggest that miR-548a-5p acts as a novel tumor promoter in HCC cells and contributes to the tumor behavior by targeting the Tg737 gene.

MiR-548 was identified as a large human gene family with 69 members, which are mainly expressed in primates and play a key role in multiple biological processes[22]. The miR-548 family possesses the most abundant primate-specific miRNA common seed (5'-AAAGUA-3') detected by computational characterization[27] and the signatures were identified to be down-regulated in peripheral blood mononuclear cells from heart failure patients. This information suggests that miR-548 level could be used as a potential biomarker specific for cardiac dysfunction[28]. In addition, endogenous miR-548 levels were suppressed during viral infections. MiR-548 shows significant identity to three viruses (HCV, HBV and HIV-1)[29], and regulated the host antiviral response *via* direct targeting interferon[30]. The marked up-regulation of miR-548ah-5p suggests that it might be related to the transition from the immune tolerance phase to the immune activation phase in chronic hepatitis B[31]. Such evidence makes miRNA a valuable therapeutic agent for patients diagnosed with multiple virus infection. Regarding the relationship of miR-548 with tumors, an analysis of the expression profiles and functional affinities of 3500 putative target genes of miR-548 suggested its cancer-related regulatory role[32]. A recent study found that miR-548d-3p, another miR-548 family member, is usually overexpressed in numerous types of cancer and affects tumorigenesis[33]. Overexpression of the miR-548 family, especially miR-548a-5p and miRNA-548d-5p, could play a complementary role in supporting the oncogenicity in cervical carcinogenesis[34]. Similar results were obtained in our study. MiR-548a-5p

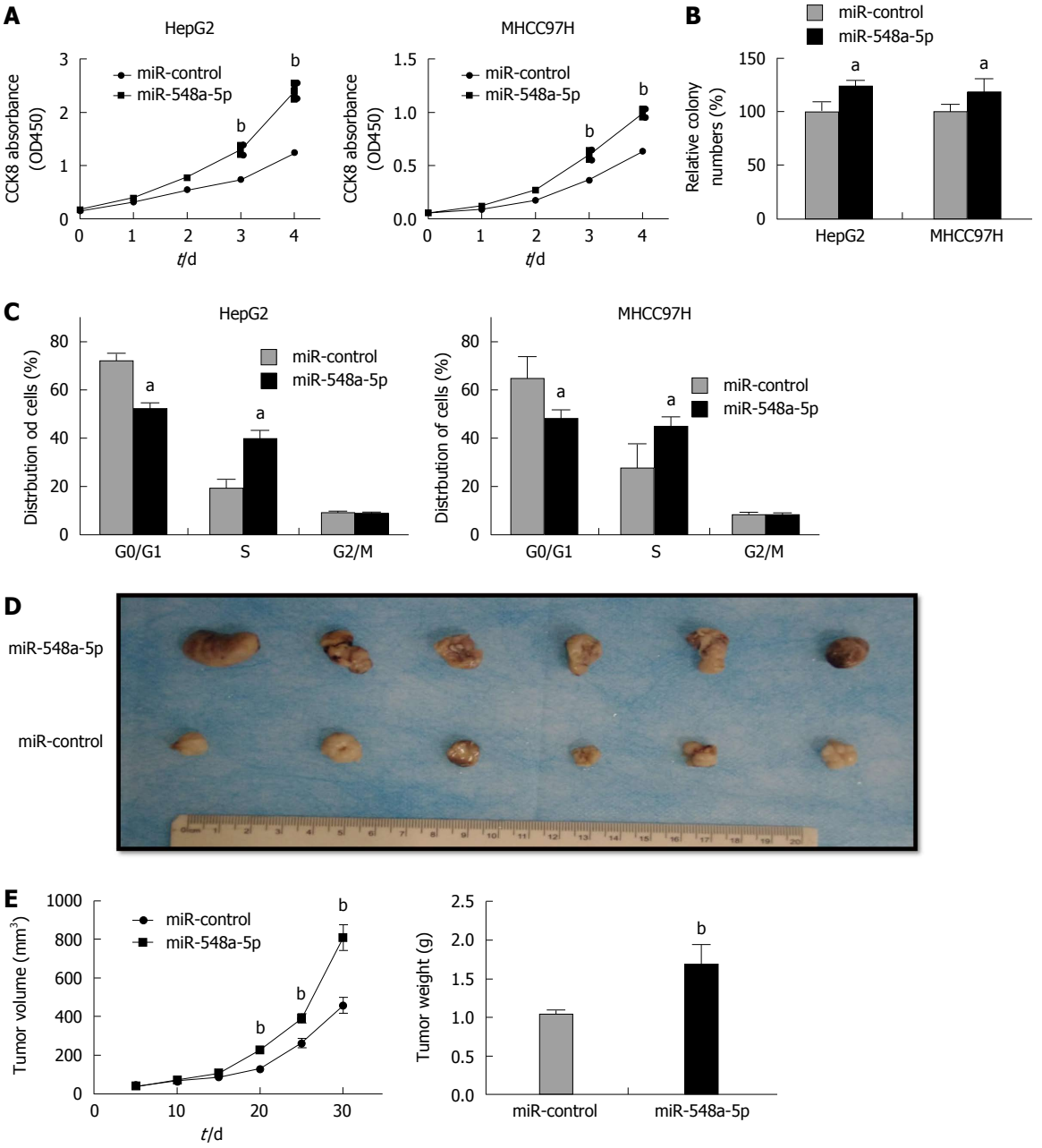

Figure 3 miR-548a-5p promotes hepatocellular carcinoma cell proliferation *in vitro* and *in vivo*. A: CCK-8 assays of HepG2 and MHCC97H cells constantly expressing miR-548a-5p or miR-control; B: Colony forming assays of HepG2 and MHCC97H cells constantly expressing miR-548a-5p or miR-control; C: Cell cycle assays of HepG2 and MHCC97H cells constantly expressing miR-548a-5p or miR-control; D: MiR-548a-5p or miR-control overexpressing HepG2 cells were injected into the subcutaneous layer of nude mice. The mice were killed 30 d after injection; E: Tumor volumes and weight. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control.

overexpression accelerated HepG2 and MHCC97H cell proliferation *in vitro* and increased the tumor size and weight *in vivo*, which indicates that miR-548a-5p promotes hepatic neoplasia.

Tg737 has attracted much attention because its functional inactivation by regulation of its expression is relevant to the formation of many solid tumors, such as those in the liver, kidney, and pancreas[4-5]. As a liver tumor suppressor gene, it has been implicated to be important for cell proliferation and apoptosis[35], because it is found deficient in HCC samples from chemical-induced rats and clinical patients[7,36]. In our previous research, Tg737 was found to be remarkably decreased in gene expression in side population cells of HCC, and the loss of heterozygosity of Tg737 was markedly associated with a poor prognosis in patients with HCC after curative liver resection[37]. In the present study, we confirmed these results and found that a loss of Tg737 function showed growth promotion in HepG2 and MHCC97H cells, which corresponds to the results of miR-548a-5p overexpression. Furthermore, miR-548a-5p knockdown rescued the effects caused by Tg737. There was a significant inverse correlation between miR-548a-5p expression and Tg737 mRNA and protein levels in hepatocellular carcinoma tissue and cell specimens. Thus, all these

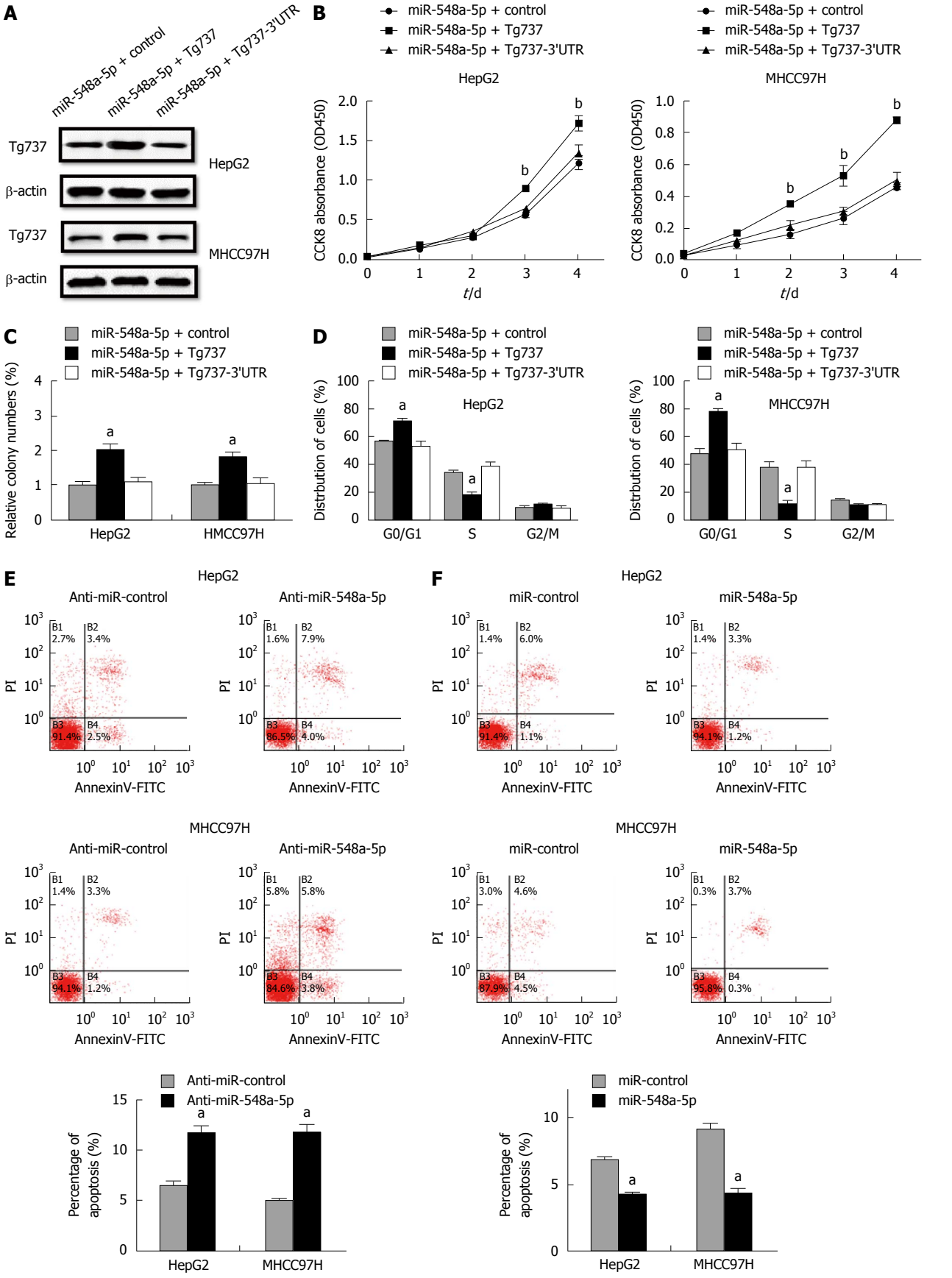

Figure 4 Restoration of Tg737 rescues the miR-548a-5p induced cell proliferation inhibition and apoptosis induced by cisplatin. A: Western blot was performed 48 h after transfection of pc CON, pc Tg737 and plasmid with 3'UTR of Tg737 into miR-548a-5p overexpressing HepG2 and MHCC97H cells; B: CCK-8 assay; C: Colony forming assay; D: Cell cycle assay; E: Cell apoptosis increased in anti-miR-548a-5p transfected HepG2 and MHCC97H cells; F: Cell apoptosis decreased in miR-548a-5p transfected HepG2 and MHCC97H cells. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control.

results support the hypothesis that miR-548a-5p functions in HCC by targeting the 3'UTR of Tg737.

In summary, our observations provide novel evidence that miR-548a-5p negatively regulates the tumor inhibitor gene Tg737 and promotes tumorigenesis *in vitro* and *in vivo*, adding to our understanding of the biological function of miR-548a-5p, as well as its relationship with Tg737. Therapeutic strategies to inhibit miR-548a-5p therefore potentially may be useful in limiting HCC growth and metastasis. A practical application of our findings could be the use of miR-548a-5p expression as a potential predictor of tumors that may be more likely to respond to Tg737-targeting therapies.

COMMENTS

Background

Primary hepatocellular carcinoma (HCC) is one of the most common and lethal malignancies. Despite advances in the surgical therapies, the reason of frequent recurrence remains mostly unclear. Several studies show that some genes may have good roles in cell proliferation and migration in HCC cells. The Tg737 gene is now identified as a tumor suppressor gene in multiple cancers, including those of the liver, kidney, and pancreas. In liver tissues from HCC patients, a 59% down-regulation of the Tg737 was observed. This gene may participate in alterations in a series of human or rodent liver tumors and tumorigenic cell lines. Nevertheless, the regulatory factor for Tg737 is not fully clear.

Research frontiers

In recent years, microRNAs (miRNAs) emerged as a group of important endogenous modulators of gene function at the posttranscriptional level. It has been revealed that many miRNAs are underscored by the promotion of tumorigenesis and cancer progression. More importantly, in HCC, several miRNAs have been demonstrated to contribute to the tumor regulation, including, but not limited to, migration, invasion, and proliferation. Multiple miRNAs have been identified as down-regulated tumor-suppressing genes involved in cellular processes, as oncogenic miRNAs, showed up-regulated expressions which potentially targeted many tumor-suppressive genes. MiR-548 is a large and poorly conserved primate-specific miRNA family. The authors previous studies have found high expression of miR-548c-3p and miR-548c-5p in side population cells from HCC. However, like the homologous gene, the exact effect of miR-548a-5p on HCC is still unknown. The direct aim of this study was to investigate whether Tg737 is regulated by miR-548a-5p, and correlates with HCC cell proliferation and apoptosis.

Innovations and breakthroughs

MiR-548a-5p negatively regulates the tumor inhibitor gene Tg737 and promotes tumorigenesis *in vitro* and *in vivo*. The novelty of the research represents the idea that the inhibition of miR-548a-5p may limit HCC growth, and miR-548a-5p expression may be the potential predictor of tumors that respond to Tg737-targeting therapies.

Applications

In summary, this observations provide novel evidence that miR-548a-5p negatively regulates the tumor inhibitor gene Tg737, and promotes tumorigenesis *in vitro* and *in vivo*, which adds to the authors understanding of the biological function of miR-548a-5p, as well as the relationship with Tg737. Therapeutic strategies to inhibit miR-548a-5p therefore potentially may be useful to limit HCC growth and metastasis. A practical application of the authors findings could be the use of miR-548a-5p expression as a potential predictor of tumors that may be more likely to respond to Tg737-targeting therapies.

Peer-review

The study by Zhao Ge *et al* on microRNA-548a-5p and its role in hepatocellular carcinoma *via* Tg737 is very interesting. The authors show that miR-548a-5p regulates negatively the tumor suppressor gene Tg737 and promotes tumorigenesis *in vitro* and *in vivo*. The authors suggest that therapeutic strategies to inhibit miR-548a-5p in the future may prove useful in limiting HCC growth and metastasis.

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ORIGINAL ARTICLE

Basic Study

Curcumin improves regulatory T cells in gut-associated lymphoid tissue of colitis mice

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Abstract

AIM: To explore the probable pathway by which curcumin (Cur) regulates the function of Treg cells by observing the expression of costimulatory molecules of dendritic cells (DCs).

METHODS: Experimental colitis was induced by administering 2, 4, 6-trinitrobenzene sulfonic acid (TNBS)/ethanol solution. Forty male C57BL/6 mice were randomly divided into four groups: normal, TNBS + Cur, TNBS + mesalazine (Mes) and TNBS groups. The mice in the TNBS + Cur and TNBS +Mes groups were treated with Cur and Mes, respectively, while those in the TNBS group were treated with physiological saline for 7 d. After treatment, the curative effect of Cur was evaluated by colonic weight, colonic length, weight index of the colon, and histological observation and score. The levels of $CD4^+CD25+Foxp3^+$ T cells (Treg cells) and costimulatory molecules of DCs were measured by flow cytometry. Also, related cytokines were analyzed by enzyme-linked immunosorbent assay.

RESULTS: Cur alleviated inflammatory injury of the colonic mucosa, decreased colonic weigh and histological score, and restored colonic length. The number of Treg cells was increased, while the secretion of TNF-α, IL-2, IL-6, IL-12 p40, IL-17 and IL-21 and the expression of costimulatory molecules (CD205, CD54 [ICAM-1], TLR4, CD252[OX40 L], CD256 [RANK] and CD254 [RANK L]) of DCs were notably inhibited in colitis mice treated with Cur.

CONCLUSION: Cur potentially modulates activation of DCs to enhance the suppressive functions of Treg cells and promote the recovery of damaged colonic mucosa in inflammatory bowel disease.

Key words: Curcumin; Regulatory T cells; Dendritic cells; Costimulatory molecules

Core tip: The low level of regulatory T (Treg) cells plays an important role in pathogenic process of inflammatory bowel disease (IBD). In our and other previous studies, curcumin (Cur) can effectively attenuate inflammation in humans and animals with colitis. However, whether Cur can improve the level of Treg cells and the pathway by which Cur regulates Treg cells are unclear. In the present study, we have shown that Cur potentially modulates activation of dendritic cells to enhance the suppressive functions of Treg cells and promote the recovery of damaged colonic mucosa in IBD.

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INTRODUCTION

Since 1995, regulatory T (Treg) cells as a particular lineage of $CD4^+$T cells have been found to play a central role in the effective control of self-tolerance and maintenance of immune homeostasis[1,2]. Insufficient quantity or dysfunction of Treg cells invariably leads to inflammatory diseases, autoimmune diseases or lymphoproliferative syndrome including inflammatory bowel disease (IBD), rheumatoid arthritis and systemic lupus erythematosus in humans and animals[3-6]. Many previous studies have demonstrated that the suppressive function of Treg cells limits convincingly favorable host effector responses and restrains inflammatory responses in diverse anatomical locations as mucosal barriers against chronic inflammations and tumors[7-10]. Distinct Treg subsets coexist in the intestinal mucosa and mesenteric lymph nodes. Some studies have demonstrated that the suppressive function of Treg cells is partly implemented *via* modulation of dendritic cells (DCs)[11-13]. Usually, the transfer of Treg cells leads to the development of colitis *via* accumulation of T cells and DCs[14]. However, adequate Tregs or transfusion of Treg cells maintains mucosal tolerance to prevent and cure experimental colitis by directly inhibiting the expression of costimulatory molecules (such as CD40, CD154, CD134 and CD134L) of DCs or the migration of DCs to the MLNs, or by reducing DC activation[15,16]. These findings indicate that the interactions of DCs and Treg cells are closely related to the pathogenesis of IBD, and probably become a target for treatment of IBD.

Curcumin (Cur), a major active component of the rhizome of Curcuma longa (turmeric), has been used widely to treat cardiovascular disease, diabetes mellitus and IBD for several centuries in China[17,18]. Cur is known for its low toxicity and a wide range of reported pharmacological effects, which include antioxidant, anti-inflammatory, antiplatelet, hypoglycemic, cholesterol-lowering, anti-bacterial, wound-healing and anti-fungal effects[19-21]. Several studies have demonstrated that Cur can effectively attenuate inflammation in humans and animals with colitis[19-21]. Although anti-inflammatory actions of Cur in IBD may be associated with the inhibition of nuclear factor κB (NF-κB) pathway, such as p38 mitogen-activated protein kinases, and the reduction of pro-inflammatory cytokine response[22,23], its exact mechanisms remain unclear. In the present study, we explored the pathway by which Cur regulates function of Treg cells by observing the expression of costimulatory molecules of DCs in an animal model of colitis.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice (9-12-wk-old) were purchased from the Animal Center of Peking University Health Science Center (Animal certificate number: SCXK 2012-0001). They were housed at 23 ± 2 ℃ with a humidity of 50% ± 5% in a 12 h light/dark cycle and provided with standard diet and water ad libitum. Mice

were randomly divided into four groups: normal, TNBS, TNBS + Cur, and TNBS + mesalazine (Mes) groups. Each group contained ten mice. The experimental protocol (JZ2015-016) was approved by Institutional Animal Care and Use Committee of Jiangxi University of Traditional Chinese Medicine.

Induction of colitis

Colitis was induced with 2, 4, 6-trinitrobenzene sulfonic acid (TNBS; batch number: p2297, Sigma, United States) as described by previous studies[24,25]. Except for mice in the normal group, mice in other groups were mildly anesthetized with pentobarbital sodium (40 mg/kg) *via* intraperitoneal injection before 100 mg/kg TNBS dissolved in 0.3 mL of 50% ethanol was instilled into the colon approximately 2 cm to the anus. Equal volume of physiological saline was given in the normal group.

Treatment protocol

Twenty-four hours after colitis was induced, the mice in the TNBS+Cur and TNBS+Mes groups were, respectively, administered with Cur (200 mg/kg) (purity > 95% by HPLC, batch number: GR-133-140421, GANGRUN Biotechnology, Nanjing, China) and Mes (300 mg/kg) (batch number: 130407, Sunflower Pharma Jiamusi, China) intragastrically for 7 d. The mice in the normal and TNBS groups received equal volume of physiological saline every day until the end of the experiment. All the mice were sacrificed on the 8^{th} day.

Histological evaluation

The whole colon (n = 10) was separated rapidly, and its length was measured. After clearing its contents, the colon was weighed and then divided into two parts. The weight index of the colon was calculated as colonic weight/body weight × 100%. The colonic segments were fixed in 4% paraformaldehyde solution, cut into sections, and stained with hematoxylin and eosin (HE). According to the previous study reported by Nicole and his colleagues[26], the criterion of the histological damage score (n = 10) was implemented to evaluate colonic injury. Histological score was calculated based on inflammatory cell infiltration and tissue damage. Scores for infiltration are as follows: 0: no infiltration; 1: increased number of inflammatory cells in the lamina propria; 2: inflammatory cells extending into the submucosa; and 3: transmural inflammatory cell infiltration. Scores of tissue damage are as follows: 0: no mucosal damage; 1: discrete epithelial lesions; 2: erosions or focal ulcerations; and 3: severe mucosal damage with extensive ulceration extending into the bowel wall.

Isolation of lymphocyte from gut-associated lymphoid tissue

Gut-associated lymphoid tissue (GALT) was separated and collected from the whole small intestine to the terminal rectum. GALT was triturated in 3% fetal calf serum (FCS)/PBS solution on ice cake, and filtrated *via* 300 section stainless steel cell cribble. The cell suspensions were centrifuged at 1500 rpm/min at 4 ℃ for 5 min and suspended in 3% FCS/PBS solution at 1 × 10^6-1 × 10^7/mL.

Assay of $CD4^+CD25^+Foxp3^+$ T cells by flow cytometry

The cell suspensions (n = 8) were incubated for 30 min with V450 -anti-mouse $CD4^+$Ab (0.125 μg/100 μL, BD Bioscience) and PerCP/Cy5.5 anti-mouse $CD25^+$Ab (0.25 μg/100 μL, Biolegend) at 37 ℃ in the dark. Cells were centrifuged at 1500 rpm/min and 4 ℃ for 5 min, fixed in Fix/Perm Buffer (eBioscience, San Diego, CA) for at least 1 h at 37 ℃, and then incubated with APC -anti-mouse $Foxp3^+$Ab (0.5 μg/100 μL; eBioscience) for 30 min at 37 ℃ in the dark. Cells labeled with PE rat IgG2a were used as the isotype negative control. Fluorescence-activated cell sorting analysis was performed on a FACSCalibur (BD Biosciences).

Measurement of costimulatory molecules by flow cytometry

Data acquisition was performed using a flow cytometer (FACSCalibur, BD-Pharmingen, San Diego, CA, United States) by collecting a minimum of 10000 events per sample. The frequency of positive cells was analyzed using the program Cell Quest in two regions. The lymphocyte region was determined using granularity (SSC) and size (FSC) plot. DCs were identified as a MHC^+ [$CD205^+$, CD54 (ICAM-1)$^+$, $TLR4^+$, CD252 (OX40 L)$^+$, CD256 (RANK)$^+$ and CD254 (RANK L)$^+$] population, and within this gate the $CD11c^+$ population was assessed. The following mAbs were used: APC/Cy7 anti-mouse CD11c, PerCP/Cy5.5 anti-mouse CD205, FITC anti-mouse CD54, PerCP-Cy™5.5 anti-mouse I-A/I-E (MHC-II), PE-cyanine7 anti-mouse TLR4, APC anti-mouse CD252, PE anti-mouse CD256 and PE rat anti-mouse CD254 (eBioscience, San Diego, CA). Limits for the quadrant markers were always set based on negative populations and isotype controls.

Enzyme-linked immunosorbent assay

The colonic mucosa was separated from all mice to prepare tissue homogenate. After centrifugation at 5000 rpm/min for 10 min, the supernatant was collected and used for testing the levels of cytokines by enzyme-linked immunosorbent assay (ELISA). The levels of TNF-α, IL-2, IL-6, IL-12 p40, IL-17 and IL-21 (n = 8) were determined using commercial ELISA kits according to the manufacturer's instructions (eBioscience, San Diego, CA).

Statistical analysis

Data are expressed as mean ± SE. The statistical significance was evaluated by one-way analysis of

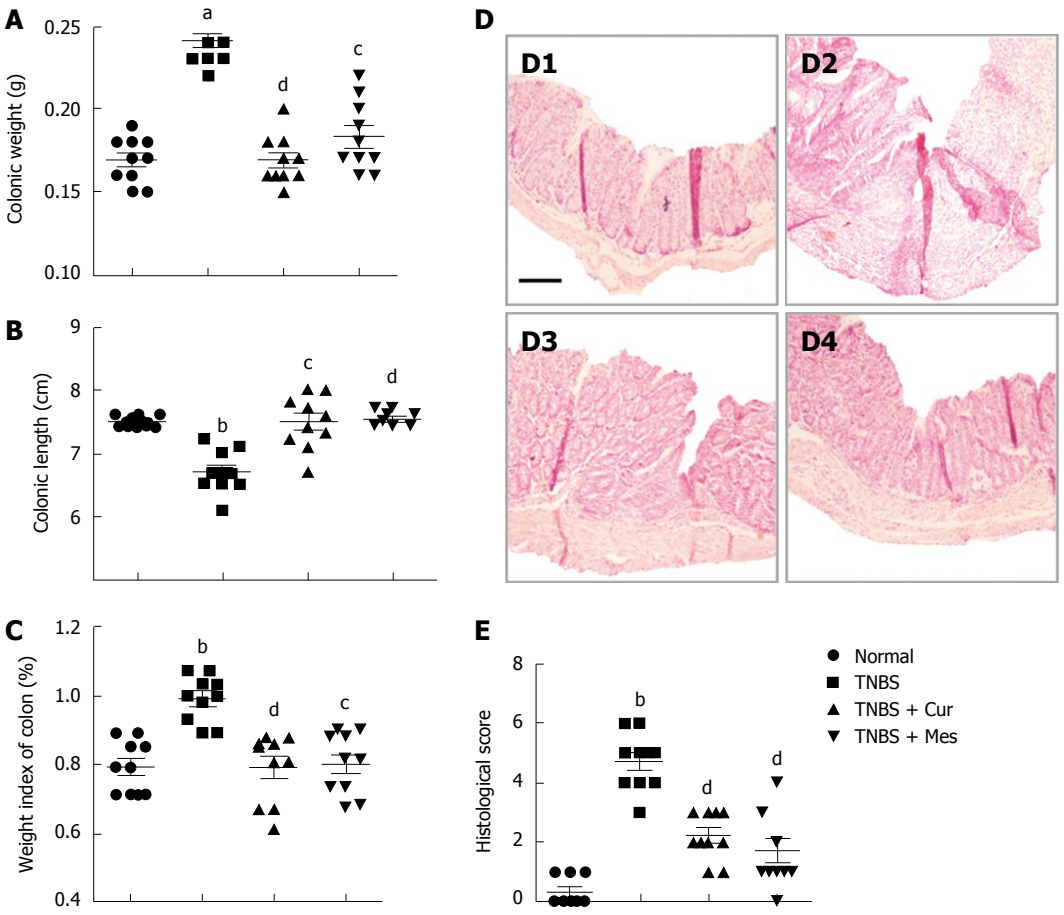

Figure 1 Histological evaluation. A: Colonic weight; B: Colonic length; C: Weight index of the colon; D: Typical histological images stained with HE. D1, D2, D3 and D4, respectively, represent the normal, TNBS, TNBS + Cur and TNBS + Mes groups. Bar = 200 μm; E: Histological scores. Data are presented as mean ± SE (n = 10). $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* normal group; $^{c}P < 0.05$ and $^{d}P < 0.01$ *vs* TNBS group.

variance, and statistical analyses were performed with Prism 4.0 (GraphPad Software, La Jolla, CA). P values less than 0.05 were considered statistically significant.

RESULTS

Cur effectively alleviates inflammatory injury of the colonic mucosa in colitis mice

Administration of TNBS led to a severe illness that was characterized by damaged colonic mucosa, increased colonic weight and so on. As shown in Figure 1, compared with mice in the TNBS group, the colonic weight (Figure 1A) and weight index of the colon (Figure 1C) were significantly decreased in the TNBS + Cur and TNBS + Mes groups, while the colonic length (Figure 1B) was lengthened. Pathological observation found that mucosal architecture was damaged, the colon wall was thicken, ulcers formed, and extensive inflammatory cells infiltrated in the colonic mucosa of colitis mice, while its histological score was increased (Figure 1D-2 and E). However, the extent of damaged colonic mucosa was alleviated in the TNBS + Cur and TNBS + Mes groups (Figure 1D-3 and D-4) as revealed by decreased histological scores (Figure 1E). These results suggest that Cur alleviates inflammatory injury of the colonic mucosa effectively.

Cur improves levels of Treg cells in GALT

As shown in Figure 2, the total number of $CD4^+$ T cells in GALT decreased in the normal, TNBS + Cur, and TNBS + Mes groups compared with the TNBS group. However, the number of $CD4^+CD25^+Foxp3^+$ T cells (Treg cells), which is a marker of Treg cells, increased in the three groups compared with the TNBS group.

Cur inhibits secretion of related cytokines in the colonic mucosa of mice with colitis

Significant increases in the secretions of TNF-α, IL-2, IL-6, IL-12 p40, IL-17, and IL-21, as assessed by ELISA in Figure 3, were observed in the TNBS group compared to the normal group. Notably, the increased expression of TNF-α, IL-2, IL-6, IL-12 p40, IL-17, and IL-21 was remarkably reduced in the TNBS + Cur group after treatment with Cur compared to the TNBS group.

Cur reduces expression of costimulatory molecules in GALT

Figure 3 shows the expression of CD205 (Figure 4A), CD54 (ICAM-1) (Figure 4B), TLR4 (Figure 4C), CD252

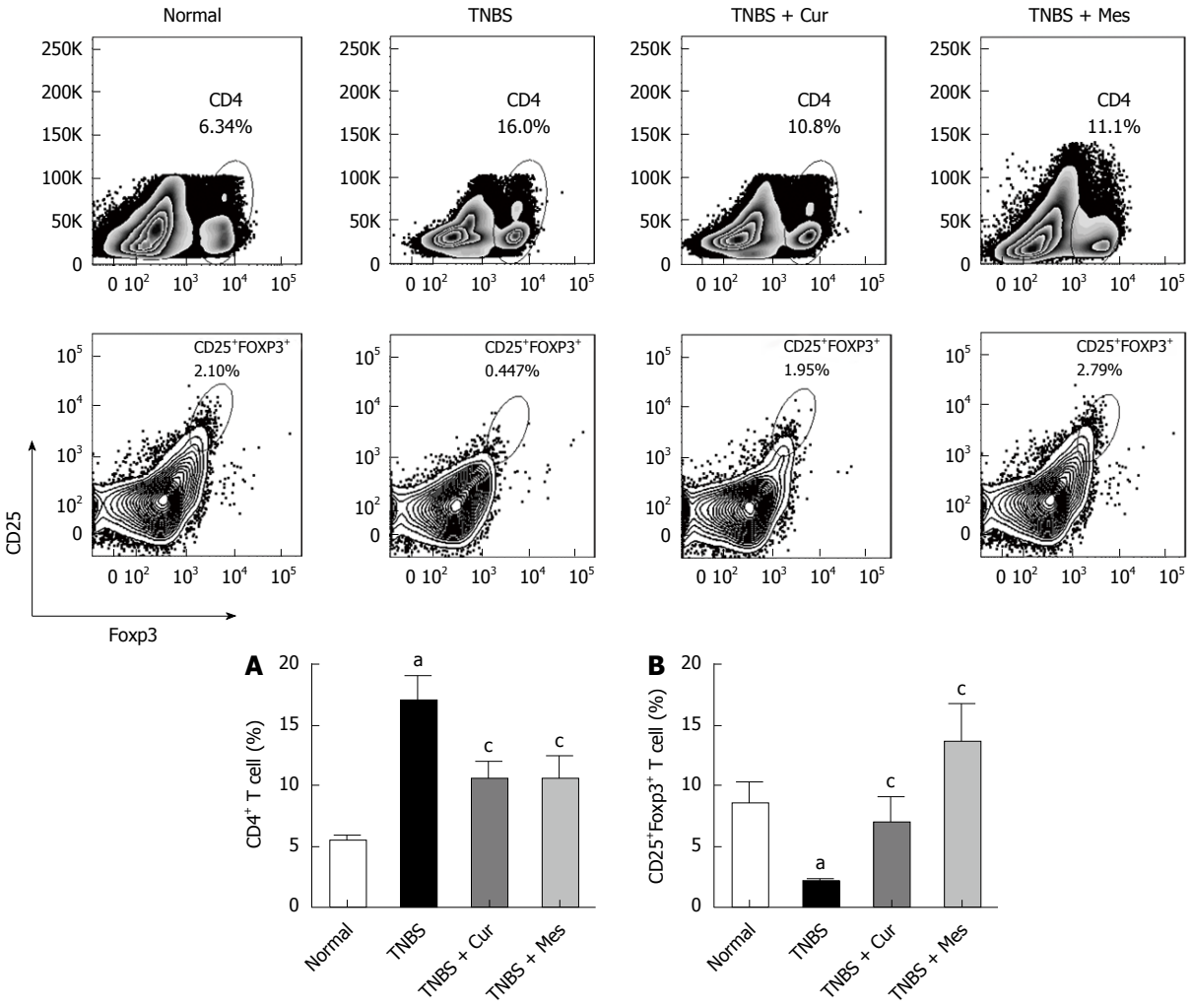

Figure 2 Typical graphs and levels of CD4$^+$ and CD25$^+$Foxp3$^+$ T cells. Lymphocytes were isolated from GALT of normal mice or mice with TNBS-induced colitis, and analyzed by flow cytometry. A: Typical graphs and levels (MFI) of CD4$^+$ T cells; B: Typical graphs and levels (MFI) of CD25$^+$Foxp3$^+$ T cells. Data are expressed as mean ± SE (n = 8). $^aP < 0.05$ *vs* normal group; $^cP < 0.05$ *vs* TNBS group.

(OX40 L) (Figure 4D), CD256 (RANK) (Figure 4E) and CD254 (RANK L) (Figure 4F) in GALT. The expression of these costimulatory molecules was increased in the TNBS group compared with the normal group. Interestingly, after 7 d treatment the increased levels of costimulatory molecules were coincidently inhibited or down-regulated in the TNBS + Cur and TNBS + Mes groups compared with the TNBS group.

DISCUSSION

Many studies have indicated that IBD is usually characterized by inflammatory injury of the colonic mucosa and disorder immune responses in inflamed mucosa, along with the dominance of IL-17-producing cells and deficiency of Treg cells[27-29]. In the present study, the results showed that Cur repaired colonic structure, decreased colonic weigh and histological injury score, recovered colonic length, indicating that Cur effectively restored damaged colonic mucosa in mice with TNBS-induced colitis. The overexpression of IL-17 and decreased Treg cells in the development of TNBS-induced colitis in our experiments were in agreement with those previous studies. After treatment with Cur, the level of IL-17 decreased, and Treg cells increased, indicating that the protective effect of Cur against TNBS-induced colitis is closely associated with decreased IL-17 expression and recovered levels of Treg cells. Also, we found that the production of related cytokines (as TNF-α, IL-2, IL-6, IL-12 p40 and IL-21) and expression of costimulatory molecules were suppressed by treatment with Cur. It is known that these inhibitory cytokines are secreted by DCs. Therefore, the effect of Cur in experimental colitis is closely with the suppressive function of Treg cells and activation of DCs.

Treg cells, also known as CD4$^+$CD25$^+$Foxp3$^+$T cells, are involved in the maintenance of peripheral tolerance and control of the immune response by initiating suppressive effects on activated immune cells[30,31]. Treg-mediated suppression can be regulated primarily by the four broad categories of mechanisms including suppression by inhibitory cytokines (IL-10, IL-35 and TGF-β), cytolysis, metabolic disruption, or

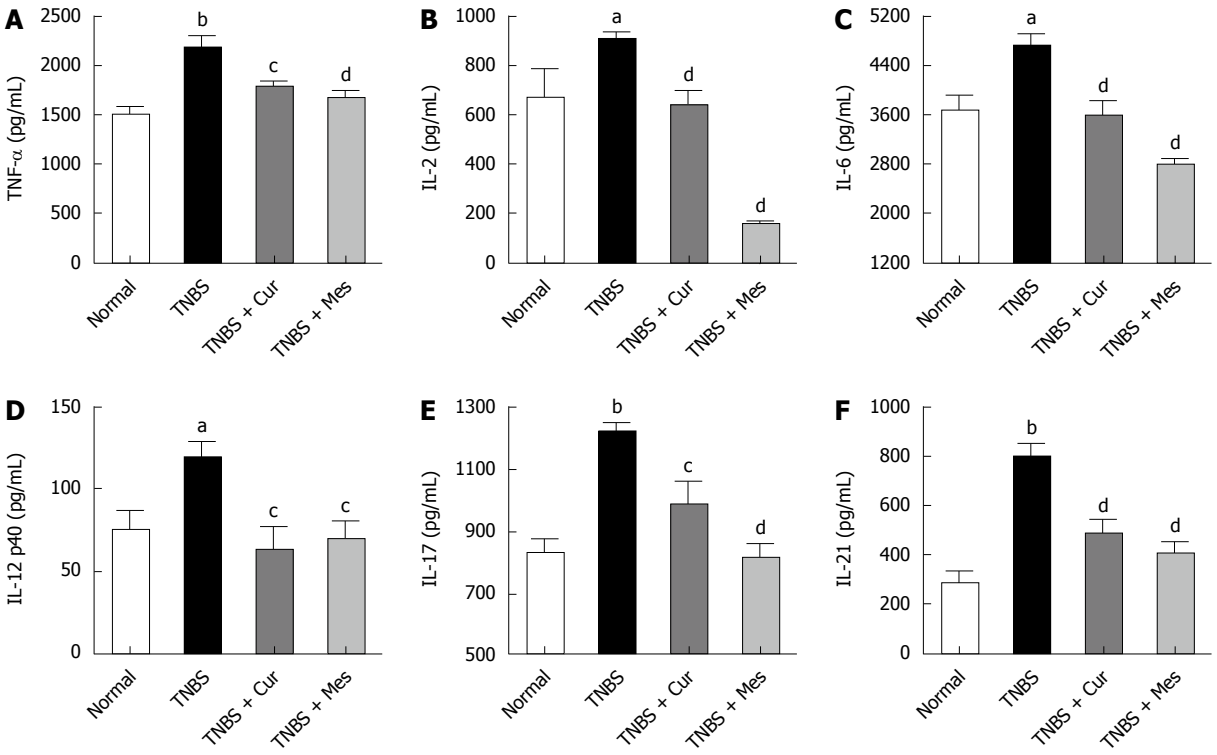

Figure 3 Concentrations of cytokines in the colonic mucosa. The tissue supernatant was prepared from the colonic mucosa in all mice. The levels of cytokines were analyzed by ELISA. A: Concentrations of TNF-α in the colonic mucosa from different groups; B: Concentrations of IL-2 in the colonic mucosa from different groups; C: Concentrations of IL-6 in the colonic mucosa from different groups; D: Concentrations of IL-12 p40 in the colonic mucosa from different groups; E: Concentrations of IL-17 in the colonic mucosa from different groups; F: Concentrations of IL-21 in the colonic mucosa from different groups. Data are expressed as mean ± SE (n = 8). $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* normal group; $^{c}P < 0.05$ and $^{d}P < 0.01$ *vs* TNBS group.

modulation of DC maturation or function[11-13]. One of mechanisms of Treg-mediated suppression is realized by anti-inflammatory cytokines (IL-10, IL-35) and TGF-β, which are in balance with pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, IL-12, and IL-21. These pro-inflammatory cytokines are inhibited by anti-inflammatory cytokines, and as a result, Th17 cells are promoted to secrete IL-17 and eventually induce inflammatory damage. IL-17 is overwhelmingly produced by Th17 cells[12,32], and is expressed extensively in the mucosa and serum of IBD patients and TNBS-induced colitis mice[33]. This suggests that the balance of Treg cells and Th17 cells was possibly broken in GALT of mice with colitis.

Foxp3, a forkhead/winged helix transcription factor, can maintain the generation, function, and stabilization of Treg cells[12]. Hence, the level of Treg cells is usually evaluated by the expression of Foxp3 in the nucleus of Treg cells. High expression of Foxp 3 is essential for effector cytokines of T helper (Th) 1, Th2, and Th17 lineages[34,35]. For example, IL-2 can maintain the stable Foxp3 expression in Treg cells *via* STAT5 phosphorylation[36,37]. Moreover, IL-6 may cause Treg cells to transfer into Th17 cells and secrete abundant IL-17 and IL-21. IL-21 combined with IL-23 induces signal transducer and activator of transcription-3 expression, promotes retinoid-related orphan receptor-γt activation, and finally improves secretion of IL-17[38-40]. In our study, down-regulation of Treg cells led to an imbalance of Treg and Th17 cells and also an imbalance of Th1 and Th2 cells, and thus induced strong expression of pro-inflammatory cytokines that resulted in inflammatory damages in the colonic mucosa.

As the uppermost part, DCs can modulate Treg-mediated suppressive function *via* expression of costimulatory molecules[41,42]. DCs are critical for regulation of intestinal immunity and mucosal immune tolerance to commensal microorganisms, which is one of the pivotal etiologies of IBD[41]. Maturation and migration of DCs in lymph nodes are "danger" signals that induce inflammatory injuries in the intestinal mucosa[42,43]. Mature DCs with expression of costimulatory molecules can regulate the balance between Th1 and Th2 cells, and the balance of Treg and Th17 cells. Misra and colleagues have demonstrated that low expression of costimulatory molecules of DCs can induce immature and limited antigen presentation of DCs, which will ineffectively stimulate T cell responses and then induce an increase in the number of Treg cells[44]. Certainly, Treg cells can secrete inhibitory cytokines to repress activation of MHC-II and costimulatory molecules of DCs *via* negative feedback to suppress the antigen presentation of DCs[45,46]. Furthermore, DCs can induce the differentiation of Th17 cells to regulate the balance of Treg cells and Th17 cells by secreting IL-6 or IL-12 p40 and producing more pro-inflammatory cytokines (such

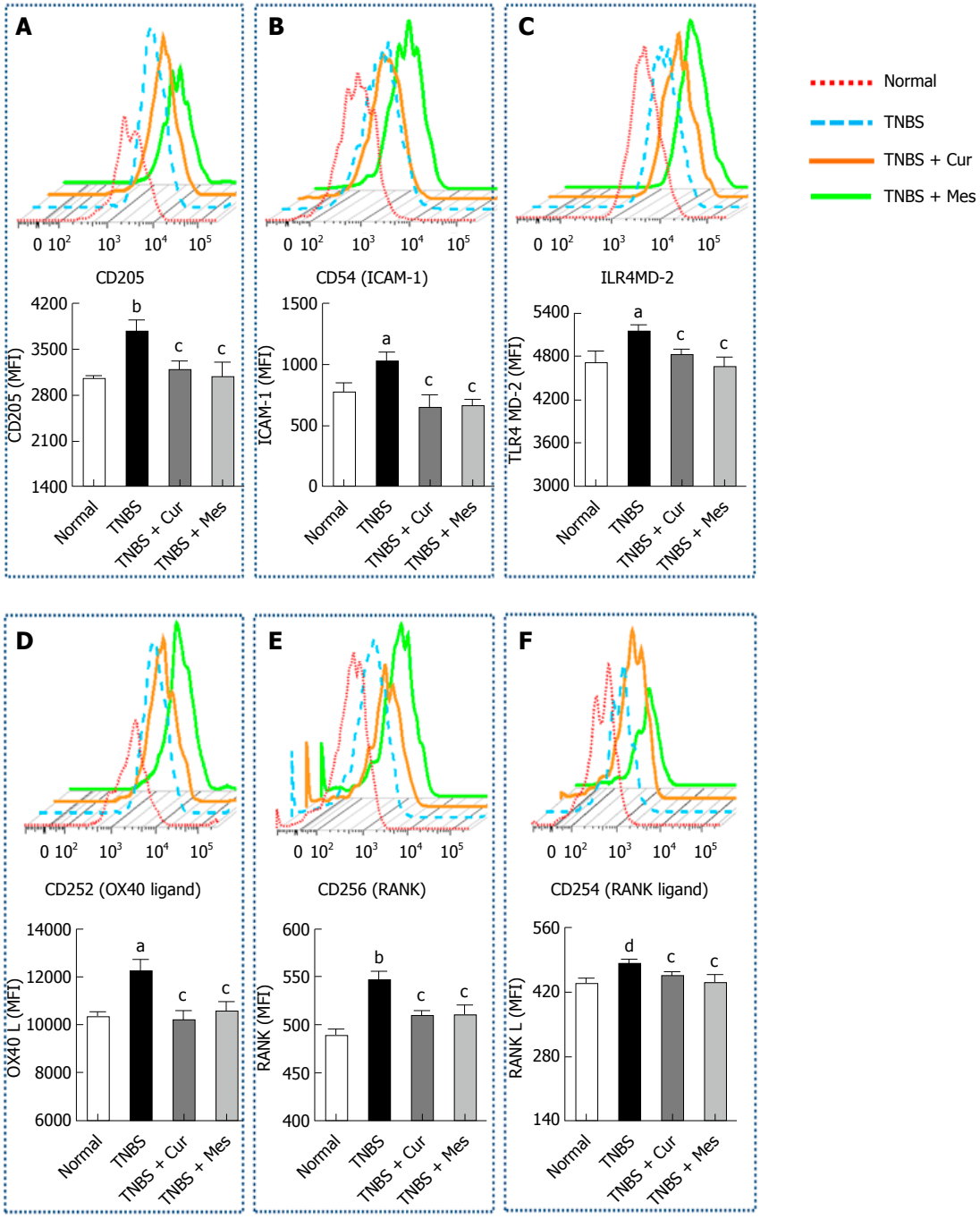

Figure 4 Typical curves and levels of costimulatory molecules in dendritic cells. Lymphocytes were isolated from GALT of normal mice or mice with TNBS-induced colitis, and analyzed by flow cytometry. A: Typical pseudocolor and level (MFI) of CD205; B: Typical pseudocolor and level (MFI) of CD54(ICAM-1); C:Typical pseudocolor and level (MFI) of TLR4; D: Typical pseudocolor and level (MFI) of CD252 (OX40 L); E: Typical pseudocolor and level (MFI) of CD256 (RNAK); F: Typical pseudocolor and level (MFI) of CD254 (RNAK L). Data are expressed as mean ± SE (n = 8). $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* normal group; $^{c}P < 0.05$ and $^{d}P < 0.01$ *vs* TNBS group.

as TNF-α, IL-6, and IL-15) that mediate inflammatory injury[47,48]. Meanwhile, costimulatory molecules of DCs, which include TNF/TNF receptor protein families (CD40/CD40L, OX40/OX40L, TNFR/TNF and so on) and immune globulin superfamily (ICAM-1/LAF-1, CD28/CTLA4/B7, *etc.*), participate in the polarization of Th1 and Th2 cell responses. As an example, receptor activator of NF-κB (RANK)/receptor activator of NF-κB ligand (RANKL) signal is an important costimulatory factor that activates DCs and prolongs their lifespan. This is achieved by activating *Bcl-XL* gene and cooperating with other costimulatory molecules (ICAM-1, TLR4, OX40 L, *etc.*) and some cytokines (IL-6 and IL-12) to activate NF-κB and induce the secretion of pro-inflammatory cytokines (TNF-α, IL-6, IL-17, and IL-21)[49]. The ICAM-1/LFA-1 signal and B7-1 molecule (B7/CD28 signal) can activate DCs to induce Th1 cell response *via* secretion of IL-12. However,

B7-2 molecule and OX40/OX40L signal promote the polarization of Th2 cells. These costimulatory molecules of DCs were highly expressed in human and animal colitis[50,51]. Thus, DCs are closely related to the development of IBD, suppression of Treg cells and balance of Treg/Th17 cells. In the present study, Cur noticeably inhibited the expression of costimulatory molecules of DCs that ineffectively stimulated T cell response to increase the suppression or number of Treg cells, and maintain Treg-mediated suppression. Also, Cur down-regulated the secretion of cytokines including IL-6 and IL-12 p40 by inhibiting DCs to prevent Treg cells from transferring into Th17 cells, inhibit the production of pro-inflammatory cytokines (TNF-α, IL-2, and IL-6) and decrease the destructive effects of IL-17 and IL-21. The mechanisms by which Cur maintains Treg-mediated suppression were only partly revealed in the present study. Therefore, further study is needed to determine whether cytolysis of Treg cells and metabolic disruption of Treg cells are also mechanisms through which Cur maintains Treg-mediated suppression.

In conclusion, Cur potentially modulates activation of DCs to enhance the suppressive functions of Treg cells and promote the recovery of damaged colonic mucosa in IBD.

COMMENTS

Background

Regulatory T cells (Treg) play a crucial role in the maintenance of self tolerance and the prevention of inflammatory bowel disease. Treg-mediated suppression can be implemented primarily by the four broad categories of mechanisms including suppression by inhibitory cytokine, cytolysis, metabolic disruption, or *via* modulation of dendritic cells (DCs) maturation or function. In these four facts, the activation of DCs is thought as the main role to maintain the suppression of Treg cells.

Research frontiers

The mature DCs with an expression of costimulatory molecules can regulate the balance between balance of Treg and Th17 cells. The previous had demonstrated that higher level expression of costimulatory molecules of DCs can lead to a decreased numbers of Treg cells to induce IBD.

Innovations and breakthroughs

The present study is firstly shown that curcumin (Cur) potentially modulated activation of DCs to enhance the suppressive functions of Treg cells and restore damaged colonic mucosa of inflammatory bowel disease (IBD).

Applications

It is known that Cur effectively treated experimental colitis by many pathway including the inhibition of nuclear factor κB pathway and reduction of pro-inflammatory cytokine response. However the level and pathway are ambiguous that Cur can or not regulate Treg cell. In the present study, our results had hinted that Cur can height the suppressive function of Treg cells *via* inhibiting the activation of DCs. The results are favorable to explore the mechanism of Cur treated chronic colitis.

Terminology

These costimulatory molecules of DCs, which include TNF/TNF receptor protein families (CD40/CD40L, OX40/OX40L, TNFR/TNF and so on) and immune globulin superfamily (ICAM-1/LAF-1, CD28/CTLA4/B7, *etc.*), are marker of activation of DCs, and decreased to inhibit the suppressive function of Treg cells in many previous documents .

Peer-review

This is a well-designed and well-presented study for examining the anti-inflammatory potential of curcumine in TNBS colitis of mice. The authors found that curcumine modulates the action of dendritic cells to enhance suppressive Treg functions, leading to an accelerated mucosal healing.

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ORIGINAL ARTICLE

Case Control Study

Adjuvant sorafenib after heptectomy for Barcelona Clinic Liver Cancer-stage C hepatocellular carcinoma patients

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Informed consent statement: Written informed consent was obtained from all patients for their data to be used for research purposes.

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Abstract

AIM: To investigate the efficacy and safety of adjuvant sorafenib after curative resection for patients with Barcelona Clinic Liver Cancer (BCLC)-stage C hepatocellular carcinoma (HCC).

METHODS: Thirty-four HCC patients, classified as BCLC-stage C, received adjuvant sorafenib for high-risk of tumor recurrence after curative hepatectomy at a tertiary care university hospital. The study group was compared with a case-matched control group of 68 patients who received curative hepatectomy for HCC during the study period in a 1:2 ratio.

RESULTS: The tumor recurrence rate was markedly lower in the sorafenib group (15/34, 44.1%) than in the control group (51/68, 75%, P = 0.002). The median disease-free survival was 12 mo in the study group and 10 mo in the control group. Tumor number more than 3, macrovascular invasion, hilar lymph nodes metastasis, and treatment with sorafenib were

significant factors of disease-free survival by univariate analysis. Tumor number more than 3 and treatment with sorafenib were significant risk factors of disease-free survival by multivariate analysis in the Cox proportional hazards model. The disease-free survival and cumulative overall survival in the study group were significantly better than in the control group (P = 0.034 and 0.016, respectively).

CONCLUSION: Our study verifies the potential benefit and safety of adjuvant sorafenib for both decreasing HCC recurrence and extending disease-free and overall survival rates for patients with BCLC-stage C HCC after curative resection.

Key words: Hepatectomy; Hepatocellular carcinoma; Sorafenib; Survival; Tumor recurrence

Core tip: Hepatocellular carcinoma (HCC) has a 5-year recurrence rate reaching 80%-90% even after potentially curative treatment. Therefore, it is extremely important to prevent recurrence for the prognosis of HCC patients. Sorafenib is the only approved treatment for patients with advanced HCC. Thus, based on the action of sorafenib, namely inhibition of tumor cell proliferation and angiogenesis, there is rationale for the study of sorafenib as an adjuvant therapy in HCC. It is essential to investigate the efficacy and safety of adjuvant sorafenib administered following curative resection in patients with Barcelona Clinic Liver Cancer-stage C HCC.

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INTRODUCTION

The incidence of hepatocellular carcinoma (HCC) is increasing globally in conjunction with increasing prevalence of hepatitis C virus infection in the West and of both hepatitis B and C in Asia[1]. Surgical resection remains the mainstay of treatment for HCC, according to guidelines and consensus coming from all over the world[2-4]. Most HCC patients are in the advanced stage of disease on initial diagnosis[5,6]. According to the Barcelona Clinic Liver Cancer (BCLC) staging system, surgical resection should only be used on a very small population of patients with very early staged HCC, while sorafenib, a multi-kinase inhibitor, is recommended as the treatment for advanced HCC. However, patients with advanced HCC who receive sorafenib achieve a median gain in overall survival (OS) of around 3 mo[7-9]. Therefore, additional management strategies need to be identified to improve therapeutic benefits. Hepatectomy for advanced HCC patients remains controversial, although previous studies showed survival benefit in patients with more advanced HCC[10]. However, early tumor recurrence is common after hepatectomy for HCC with macrovascular invasion, multinodular or large HCC. There is still no acknowledged adjuvant therapy for HCC after radical surgery, and tumor recurrence is still the main cause of death for these patients. It has been reported that sorafenib can be used as an adjuvant therapy to reduce the risk of recurrence after potentially curative treatment in animal research and in pilot clinical studies[10-13]. Feng *et al*[12] reported that sorafenib suppressed development of postsurgical intrahepatic recurrence and abdominal metastasis, which consequently led to prolonged postoperative survival, in an orthotopic xenograft model of HCC. Saab *et al*[13] demonstrated the safety and potential benefit of sorafenib, in both reducing HCC recurrence and extending disease-free survival (DFS) and OS rates, in their study of eight high-risk liver transplant recipients. Thus, further research on the efficacy of sorafenib in decreasing HCC recurrence after liver resection in high-risk patients is urgently needed. In this study, sorafenib was used as an adjuvant therapy to decrease the risk of tumor recurrence following potentially curative resection in patients with HCC of BCLC-stage C. The efficacy and safety of this modality were evaluated in this case-matched comparative study.

MATERIALS AND METHODS

Patient selection

From September 2010 to September 2013, 41 HCC patients classified as BCLC-stage C received adjuvant sorafenib after curative liver resection. This is a retrospective study on data that were prospectively collected and entered into a computer database. All patients were histologically confirmed to have HCC in the resected specimens. The definition of BCLC-stage C was: any tumor with radiological and histological evidence of macrovascular invasion (portal vein, hepatic vein, inferior vena cava). Patients with lymph nodes (Hilar lymph nodes excluded) and/or distant metastases[2,8], tumor recurrence diagnosed within 2 mo of operation, or who had received any other forms of adjuvant or neoadjuvant therapy were excluded. Patients in the sorafenib group were recruited if they agreed to take adjuvant sorafenib. Each patient in the study group was matched, as closely as possible, with two BCLC-stage C patients for tumor size (± 1 cm in diameter), tumor number, tumor location, type of operation and Child-Pugh grading from a prospectively maintained database of 1335 patients who received R0 liver resection for stage BCLC A/B/C HCC during the study period (the control group). The demographics,

blood biochemistry, tumor characteristics, surgical variables, length of hospital stay, and postoperative complications were compared. This study was approved by the Ethics Committee of the Southwest Hospital before the study began; it conformed to the ethical guidelines of the 1975 Helsinki Declaration. Written informed consent was obtained from all patients for their data to be used for research purposes.

Preoperative assessments

The patients were evaluated pre-operatively according to the routine procedure of our institute, as previously reported[14]. All patients underwent the same preoperative evaluation protocol that included percutaneous ultrasonography (US), spiral computed tomography (CT) of the thoraco-abdomen (from March 2010 in our institute), CT angiography of hepatic artery/hepatic vein/portal vein, electrocardiogram and blood biochemistry. Liver function was assessed by the Child-Pugh grading system (A or B) and indocyanine green clearance test at 15 min (ICG_{15}; less than 10%). The Eastern Cooperative Oncology Group performance status was 0 or 1 in this study. The liver volume to be resected and the future liver remnant volume (FRLV; more than 50% in cirrhosis and 40% in normal) were calculated using computed tomographic volumetry (UniSight, China), according to the method described by Urata *et al*[15].

Partial hepatectomy

The operation was performed as previously reported using a right subcostal or a midline incision with a right horizontal extension[16]. After excluding any unexpected intraperitoneal metastasis, intraoperative ultrasonography was used routinely to clarify the extent of tumor, detect tumor nodules in the contralateral hemiliver and invasion of tumor into major blood vessels, and to plan and mark the plane of liver transection. An intermittent Pringle's maneuver with clamp/unclamp times of 15/5 min by a tourniquet was used during liver transection using a clamp crushing technique. A low central venous pressure was applied routinely. The extent of hepatic resection was defined anatomically according to Couinaud's liver segmentation. Major hepatectomy was defined as resection of three or more liver segments. Blood transfusion was only given when the hemoglobin fell below 8.0 g/L. All patients received the same postoperative care by the same team of surgeons and were nursed in the surgical intensive care unit during the early postoperative period. Parenteral nutritional support was provided for patients with cirrhosis. All intra- and post-operative complications were recorded prospectively. Biliary leakage was diagnosed when the total bilirubin level in the drainage fluid exceeded the upper normal limit of serum bilirubin (21 μmol/L) on or after postoperative day 7.

Conventional surveillance strategy and sorafenib

After hepatectomy, patients were routinely monitored with thoraco-abdominal CT scans once every 2 mo for the first 6 mo, and then alternating between abdominal US and CT scan every 2 mo thereafter. Blood for α-fetoprotein (AFP) and hepatitis B virus (HBV) load were also checked at each of the follow-up visits. For patients with a high viral load (HBV DNA > 1×10^3 copies/mL), nucleoside analogues were given. Intrahepatic tumor recurrence was confirmed with imaging, AFP and/or histology. Whole-body positron emission tomography/computed tomography with ^{18}F-fluorodeoxyglucose (^{18}F-FDG PET/CT) was used to assess extra-hepatic metastasis. Recurrence time was calculated from the date of operation to the date of diagnosis of tumor recurrence or the date of censor of this study on June 31, 2014. The start point of survival analysis is the day of liver resection.

Sorafenib treatment would be initiated in the first month after surgery following receipt of formal consent from the patient, which was given as free choice by himself/herself. Patients in the sorafenib group were given sorafenib at a dose of 400 mg twice daily. Patients treated with sorafenib were followed-up closely for any adverse effects on an outpatient basis, with reduction in dosage or discontinuation when the drug was poorly tolerated according to the commonly used criteria for adverse events 3.0. Monitoring of complete blood count, blood biochemistry, contrast CT scan or magnetic resonance imaging were performed for evaluation of tumor recurrence every two months, similar to the control group of patients.

Statistical analysis

Statistical analyses were performed using the χ^2 test, the Fisher's exact test or the generalized linear model to compare discrete variables, and the Mann-Whitney test was used to compare continuous variables. DFS and OS rates were determined using the Kaplan-Meier method and the log-rank test. A $P < 0.05$ was considered as statistically significant. Potential factors associated with DFS after resection were analyzed using univariate and multivariate analyses. Univariate analysis was performed for 21 risk factors, and significant risk factors were subsequently analyzed by multivariate analysis using a stepwise Cox's proportional hazard regression model to identify independent prognostic factors for predicting DFS. Statistical analyses were performed with SPSS 13.0 for Windows computer software (SPSS Inc., Chicago, IL, United States).

RESULTS

The patient demographics and tumor characteristics are shown in Table 1. There were no significant differences between the two groups of patients. The median follow-

Table 1 Patient demographics and tumor characteristics

Clinical parameter	Sorafenib	Control	*P* value[2]
No. of patients	34	68	-
Males	25	50	-
Age, yr[1]	48 (21-78)	57 (18-79)	0.142
Platelet count, 10^9/L[1]	121 (31-368)	156 (30-317)	0.046
Serum albumin, g/L[1]	38 (26-52)	36 (24-51)	0.387
Serum total bilirubin, mmol/L[1]	14.6 (5.1-48.3)	16.4 (7.6-51.5)	0.283
AST, IU/L[1]	56 (11-526)	59 (12-498)	0.525
Hemoglobin, g/L[1]	12.5 (6.6-18.6)	11.5 (5.9-17.3)	0.275
AFP ⩾ 400, mg/L	23	45	0.882
Hepatitis B virus infection	29 (93.3%)	59 (92.0%)	0.692
ICG retention at 15 min, %[1]	7.7 (1.5-14.2)	7.3 (2.3-13.8)	0.482
Child-Pugh grade, A/B	27/7	54/14	-
HBV DNA, > 1/⩽ 1 × 10^5 copy/mL	26/8	49/19	0.634
Prothrombin time, s[1]	14 (10-17)	14 (10-19)	0.224
Tumor size, cm[1]	6.4 (2.8-20.2)	5.9 (2.9-21.3)	0.098
Tumor number[1]	2 (1-8)	2 (1-10)	0.187
Hilar lymph nodes metastasis, yes/no	11/23	29/39	0.315
Liver cirrhosis, yes/no	30/4	60/8	-
ECOG performance status score, PS = 0/1	31/3	61/7	0.814
Median follow-up time, mo	26	25	-

[1]Value expressed in median with range in parentheses; [2]Study group compared with control group.

Table 2 Surgical outcomes

Variable	Sorafenib	Control	*P* value[2]
Type of resection			-
Minor	8	16	
Major[3]	26	52	
Intraoperative blood loss, mL[1]	350 (50-1600)	380 (50-1800)	0.132
Intraoperative blood transfusion, mL[1]	650 (0-1500)	600 (0-1600)	0.126
No. of patients without transfusion, %	31 (75.0%)	58 (81.48%)	0.139
Postoperative AST on day 3, IU/L[1]	226 (48-896)	327 (68-1237)	0.042
Postoperative total bilirubin on day 3, mmol/L[1]	42 (19-276)	39 (18-212)	0.138
Infectious morbidity	8	12	0.566
Lung infection	3	4	
Abdominal collection	4	6	
Infection of incisional wound	1	1	
Sepsis	0	1	
Noninfectious morbidity	4	9	0.853
Pleural effusion	3	5	
Bile leak	1	4	
Liver failure	0	0	

[1]Value expressed in median with range in parentheses; [2]Study group compared with control group; [3]Major hepatectomy was defined as resection of three or more segments.

up was 26 mo and 25 mo for the sorafenib group and the control group, respectively.

Surgical variables and outcomes are shown in Table 2. Postoperative aspartate aminotransferase (AST) on day 3 was significantly lower in the sorafenib group than in the control group (P = 0.042). There were no marked differences between the two groups for the types of resection, intraoperative blood loss/transfusion and postoperative total bilirubin levels on postoperative day 3. The main complications were summarized as infectious and noninfectious morbidity. There was no in-hospital or 90-d mortality in this study. There was no significant difference in the overall morbidity rates (P = 0.853). All patients with pleural effusion and bile leakage recovered after percutaneous drainage.

Table 3 Type of recurrence

Type of recurrence	Sorafenib	Control	*P* value[1]
Hepatic	8	28	0.079
Extrahepatic	5	12	0.707
Hepatic + extrahepatic	2	11	0.142
Overall hepatic recurrence	10	39	0.008
Overall extrahepatic	7	23	0.167
Total recurrence	15 (44.1%)	51 (75.0%)	0.002

[1]Study group compared with control group.

The type of recurrence was divided into hepatic lesion and extrahepatic lesion (Table 3). The total recurrence was significantly lower in the sorafenib group (15/34, 44.1%), as compared to the control group (51/68, 75%, P = 0.002). The overall hepatic recurrence was also markedly lower in the sorafenib group (P = 0.008).

Table 4 shows the results of the univariate and multivariate analyses for the 21 selected variables. Of these variables, tumor number more than 3, presence of macrovascular invasion, positive hilar lymph nodes metastasis and treatment with sorafenib were significantly correlated to DFS. Multivariate analysis by Cox's proportional hazard model showed that tumor number more than 3 and treatment with sorafenib were independent significant risk factors of DFS.

The DFS and cumulative OS rates in the sorafenib group were significantly better than those in the control group (P = 0.034 and 0.016, respectively) (Figure 1). The median DFS was 12 mo for the sorafenib group and 10 mo for the control group. Meanwhile, the median cumulative OS was 25 mo for the sorafenib group and 18 mo for the control group.

Sorafenib was generally safe and well tolerated, with only grades 1 and 2 drug-related adverse events (14 patients, 41.2% in this study). Overall, 9 of 34 patients (26.5%) required a dosage reduction for adverse effects of sorafenib, and most patients recovered (n = 6). The remaining 3 patients were kept on half dose. The mean duration of sorafenib treatment was 22.9 mo. Viral loads were controlled satisfactorily with nucleoside analogues in most of the patients. However, 5 patients required a combination of antiviral drugs (lamivudine + adefovir dipivoxil) to control the viral load.

DISCUSSION

Since tumor recurrence remains a major obstacle

Table 4 Univariate and multivariate analyses of potential risk factors of tumor recurrence

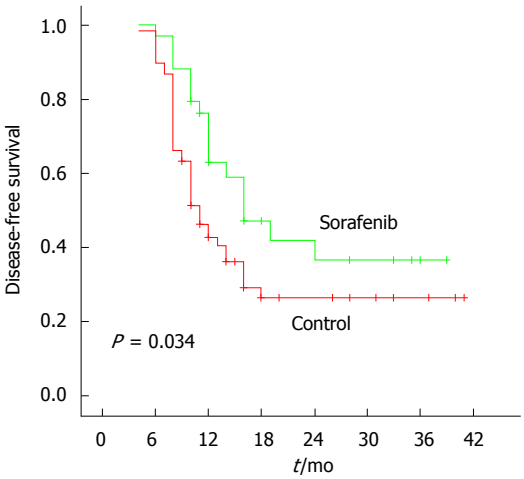
Risk factors[1]	Univariate analysis			Multivariate analysis		
	Hazard ratio	95%CI	*P* value (Cox's regression)	Hazard ratio	95%CI	*P* value (Cox's regression)
Sex, male/female	1.159	1.934-1.402	0.118			
Age, > 50/≤ 50 yr	1.003	0.961-1.012	0.751			
Platelet count, > 100/≤ 100 × 10^9/L	1.258	0.939-1.626	0.095			
Hemoglobin, > 10/≤ 10 g/L	1.119	0.867-1.411	0.089			
Serum AFP, > 400/≤ 400 ng/mL	0.960	0.929-1.011	0.079			
HBV infection, yes/no	0.931	0.593-1.449	0.769			
HBV DNA, > 1/≤ 1 × 10^5 copy/mL	1.323	0.912-1.883	0.119			
AST, > 42/≤ 42 IU/L	1.171	0.933-1.425	0.127			
Serum albumin, > 38/≤ 38 g/L	1.264	0.961-1.665	0.095			
Serum total bilirubin, > 21/≤ 21 mmol/L	0.965	0.923-1.010	0.075			
Prothrombin time, > 12.8/≤ 12.8 s	1.162	0.931-1.324	0.126			
ICG retention at 15 min, > 10%/≤ 10%	1.349	0.903-1.989	0.115			
Child-Pugh grade, A/B	1.011	0.914-1.097	0.779			
Tumor size, > 50/≤ 50 mm	0.957	0.904-1.014	0.075			
HBsAg, positive/negative	1.241	0.948-1.649	0.094			
HBeAg, positive/negative	1.174	0.938-1.422	0.121			
Type of resection, minor/major	1.131	0.949-1.381	0.086			
Tumor number, < 3/≥ 3	2.050	1.051-3.549	0.034	1.324	1.035-1.776	0.045
Macrovascular invasion, yes/no	1.242	1.061-1.501	0.035			
Hilar lymph nodes metastasis, yes/no	1.058	1.006-1.111	0.043			
Sorafenib, yes/no	1.012	1.003-1.099	0.034[a]	1.353	1.012-1.763	0.039

[1]The number of patients forin each risk factor analyzed = 102.

to good survival for HCC patients following curative resection for locally advanced HCC, an effective adjuvant therapy becomes important. Recently, sorafenib has been shown to be effective for patients with advanced HCC with Child-Pugh class A cirrhosis[9]. The present study showed that sorafenib prolonged DFS and OS by reducing tumor recurrence after curative resection for locally advanced HCC in BCLC-stage C patients.

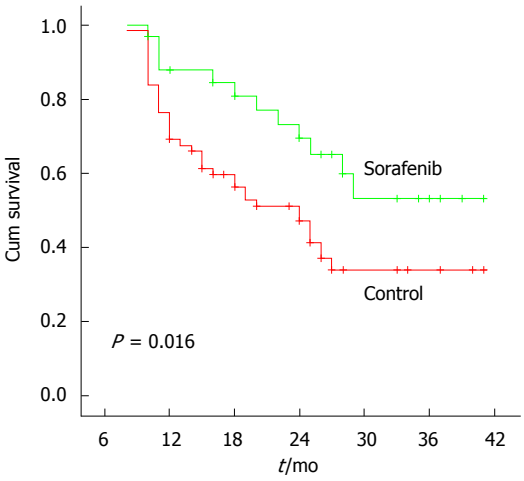
The role of liver resection for patients with locally advanced HCC has remained controversial until now. The BCLC classification has been endorsed by the European Association for the Study of Liver Disease and the American Association for the Study of Liver Disease as the best staging system and treatment algorithm for HCC[2,4]. Patients with BCLC-stage C HCC are recommended to receive sorafenib only. However, there have been many retrospective studies in which aggressive surgical therapy was shown to improve long-term survival in the selected patients with locally advanced HCC[10]. The main arguments against resectional surgery for these patients are early recurrence and metastases. Llovet *et al*[9] and Bruix *et al*[5] advocated that research efforts should be focused on adjuvant setting of resection, local ablation or combination therapy. Animal studies have indicated sorafenib inhibited tumor growth and prevented tumor recurrence after resection of HCC[12,17]. Feng *et al*[12] used a luciferase labeled orthotropic xenograft model of HCC to examine the role of sorafenib in prevention of HCC recurrence and demonstrated that sorafenib suppressed development of postsurgical intrahepatic recurrence and abdominal metastasis, which led to prolonged postoperative survival in mice. Wang *et al*[17] demonstrated that sorafenib inhibited tumor growth and prevented metastatic recurrence after resection of HCC in nude mice. There were also a few clinical reports on the effect of sorafenib in prevention of tumor recurrence[13,18]. A group from the University of California Los Angeles performed a retrospective case-matched study that included eight HCC patients who tolerated adjuvant sorafenib after orthotopic liver transplantation. This study demonstrated the safety and potential benefit of sorafenib in both reducing HCC recurrence and extending DFS and OS for high-risk liver transplant recipients[13]. Recently, a pilot study was conducted on adjuvant sorafenib for HCC patients (31 patients) who underwent curative liver surgery and had high-risk factors for tumor recurrence[18]. The time to recurrence and disease recurrence rate were assessed and showed that adjuvant sorafenib for HCC prevented early recurrence after hepatic resection. The significantly lower cumulative recurrence-free survival rate also demonstrated the preventive effectiveness of sorafenib. All these data strongly suggest that sorafenib has a potential role to play in patients with locally advanced HCC after hepatectomy with curative intention. A phase Ⅲ randomized controlled trial to evaluate whether sorafenib could be used as an effective adjuvant therapy after resection or ablation (STORM trial, NCT00692770) was reported by Bruix *et al*[19] recently. The study showed the trial did not meet its main endpoint of improving recurrence-free survival. Nevertheless, the inclusion criteria of the STORM trial was very narrow (for surgical resection: single tumor of ≥ 2 cm without microscopic vascular

A



Sorafenib						
Number of recurrence		1	11	4	2	0
Number of cumulative recurrence		1	12	16	18	18
DFS rate (%)		97.1	63.0	47.2	36.7	36.7
Control						
Number of recurrence		7	31	7	0	0
Number of cumulative recurrence		7	38	45	45	45
DFS rate (%)		89.7	42.7	26.4	26.4	26.4

B



Sorafenib						
Number of death	0	4	2	3	3	0
Number of cumulative death	0	4	6	9	12	12
OS rate (%)	100	88.0	80.9	69.3	53.2	53.2
Control						
Number of death	0	21	8	5	6	0
Number of cumulative death	0	21	29	34	40	40
OS rate (%)	100	69.1	56.3	47.2	33.8	33.8

Figure 1 Survival analysis in the sorafenib group and control group. A: Disease-free survival; B: Cumulative survival. DFS: Disease-free survival; OS: Overall survival.

invasion, without tumor satellites and histologically well- or moderately-differentiated, or any size single lesion but must be ⩾ 2 cm if well-differentiated; for local ablation: single tumor > 2 cm or ⩽ 5 cm, or 2-3 lesions with each ⩽ 3 cm in size). As all these patients were classified as BCLC early stage (A), the impact of sorafenib on these patients after curative hepatectomy may have been insufficiently robust to be demonstrated in that study. On the contrary, there is a recently published review in which a meta-analysis confirmed that the combination therapy of transcatheter arterial chemoembolization plus sorafenib in patients with intermediate or advanced stage HCC can improve OS, time to progression and objective response rate[20].

In our study, tumor number more than 3, presence of macrovascular invasion and positive hilar lymph nodes metastasis were identified as factors with the highest risk of tumor recurrence. Sorafenib was well tolerated in most of our patients. A literature review was conducted on the prognosis of BCLC-stage C HCC patients treated with either sorafenib or liver resection

Table 5 Comparison of outcomes of patients with Barcelona Clinic Liver Cancer-stage C hepatocellular carcinoma treated by various interventions

Study	Year	Number of patients	Interventions	Median OS, mo	1-, 3-, 5-yr	
					DFS, %	OS, %
Llovet *et al*[9]	2008	299	Sorafenib	10.7	-	44/-/-[1]
		303	Placebo	7.9	-	33/-/-
Cheng *et al*[22]	2009	150	Sorafenib	6.5	-	53.3/-/-[1]
		76	Placebo	4.2	-	36.7/-/-[1]
Wörns *et al*[23]	2009	22	Sorafenib	3.3	-	-
Yau *et al*[24]	2009	51	Sorafenib	5.0	-	-
Ozenne *et al*[25]	2010	50	Sorafenib	5.5	-	-
Baek *et al*[26]	2011	201	Sorafenib	5.3	-	-
Iavarone *et al*[27]	2011	296	Sorafenib	10.5[1]		
Santini *et al*[28]	2012	93	Sorafenib	12.0[1]		
Zhao *et al*[29]	2013	222	Sorafenib and TACE	12.0[1]		
Minagawa *et al*[30]	2001	18	Liver resection			82/42/42
Chirica *et al*[31]	2008	20	Liver resection	32.0	40/20/17[1]	73/56/45[1]
Ishizawa *et al*[32]	2008	98	Liver resection		-/37/25	-/71/56
Wang *et al*[33]	2008	14	Liver resection	13.0		57/29/29
Torzilli *et al*[34]	2008	28	Liver resection		66/17/-	80/74/-
Yang *et al*[35]	2012	511	Liver resection	27.8	48/30/24	70/41/31
Torzilli *et al*[10]	2013	297	Liver resection		46/28/18	76/49/38
This study	2014	68	Liver resection	18.0	42/26/-	69/34/-
		34	Liver resection and Sorafenib	25.0	63/37/-	88/53/-

[1]Including patients with BCLC-stage B. DFS: Disease-free survival; OS: Overall survival; TACE: Transarterial chemoembolization.

alone (Table 5). The median overall survival was better in the groups treated with liver resection in selected patients than with sorafenib only. In our study, when liver resection was combined with sorafenib, there was significantly better OS than for liver resection alone. To the best of our knowledge, our study is the first to evaluate adjuvant sorafenib for BCLC-stage C patients after curative resection. Adjuvant systemic treatment is the most promising of all adjuvant therapies for locally advanced HCC, as this disease is likely to be systemic at this stage.

PET/CT was used for the diagnosis of extrahepatic metastasis in this study, as ^{18}F-FDG PET/CT is currently the most sophisticated technique to detect metastases, despite the possibility of false positivity. A systematic review and meta-analysis published recently reported the pooled estimates of sensitivity, specificity, positive likelihood ratio and negative likelihood ratio of ^{18}F-FDG PET/CT in the detection of metastatic HCC were 76.6%, 98.0%, 14.68 and 0.28, respectively[21]. This study has the limitations of small case number and the study came from a single institute; also, the median follow-up was not long enough. As a retrospective study, the selection bias was fully considered and treated by matching and statistic analysis. Future prospective clinical randomized controlled trials are warranted to confirm our results.

In conclusion, this study demonstrates the safety and potential benefits of sorafenib in both decreasing the incidence of HCC recurrence and extending the DFS and OS rates for patients with locally advanced HCC after curative resection.

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COMMENTS

Background

To investigate the efficacy and safety of adjuvant sorafenib after potentially curative resection for patients with Barcelona Clinic Liver Cancer-stage C hepatocellular carcinoma (BCLC-stage C HCC).

Research frontiers

Hepatocarcinogenesis is a complex process including many signaling cascades. Currently, there is no standard of care for adjuvant therapy because no treatment has a proven benefit in randomized studies in patients with HCC after potentially curative treatment. Sorafenib is approved for use in patients with unresectable HCC based on two phase 3 randomized trials, and is the recommended treatment in patients with advanced HCC. Animal studies and a few retrospective clinical studies published recently have indicated sorafenib inhibited tumor growth and prevented tumor recurrence after resection of HCC.

Innovations and breakthroughs

Based on the mechanism of sorafenib, inhibition of tumor cell proliferation and angiogenesis, in addition to it's proven efficacy in advanced HCC, there is rationale for the study of sorafenib as an adjuvant therapy in HCC.

Applications

It is worthwhile to investigate the efficacy of adjuvant sorafenib after potentially curative resection for BCLC-stage C HCC patients with the prospective clinical randomized controlled trials in future.

Terminology

Thirty-four HCC patients received adjuvant sorafenib after curative liver resection and were compared with a case-matched control group of 68 HCC

patients who underwent hepatectomy.

Peer-review

This case-control study proves the important clinical role of sorafenib as an adjuvant treatment after R0 resection of BCLC-stage C HCC patients. It would be worth using sorafenib not just in advanced but in early stages of HCC.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Genomic change in hepatitis B virus associated with development of hepatocellular carcinoma

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Institutional review board statement: The study was reviewed and approved by the Institutional Review Board of Asan Medical Center.

Informed consent statement: Waiver of Informed Consent for this study has been granted by the Institutional Review Board of Asan Medical Center due to demonstrated minimal risk.

Conflict-of-interest statement: All the authors have no conflict of interest related to the manuscript.

Data sharing statement: All the anonymous dataset is available on request from the corresponding author at yhchung@amc.seoul.kr

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Abstract

AIM: To determine the genomic changes in hepatitis B virus (HBV) and evaluate their role in the development of hepatocellular carcinoma (HCC) in patients chronically infected with genotype C HBV.

METHODS: Two hundred and forty chronic hepatitis B (CHB) patients were subjected and followed for a median of 105 mo. HCC was diagnosed in accordance with AASLD guidelines. The whole X, S, basal core promoter (BCP), and precore regions of HBV were sequenced using the direct sequencing method.

RESULTS: All of the subjects were infected with genotype C HBV. Out of 240 CHB patients, 25 (10%) had C1653T and 33 (14%) had T1753V mutation in X region; 157 (65%) had A1762T/G1764A mutations in BCP region, 50 (21%) had G1896A mutation in precore region and 67 (28%) had pre-S deletions. HCC occurred in 6 patients (3%). The prevalence of T1753V mutation was significantly higher in patients who developed HCC than in those without HCC. The cumulative occurrence rates of HCC were 5% and 19% at 10 and 15 years, respectively, in patients with T1753V mutant, which were significantly higher than 1% and 1% in those with wild type HBV ($P < 0.001$).

CONCLUSION: The presence of T1753V mutation in HBV X-gene significantly increases the risk of HCC development in patients chronically infected with genotype C HBV.

Key words: Hepatocellular carcinoma; Chronic hepatitis B; Genomic change; Hepatitis B virus; Genotype C

Core tip: In the present study, we determined the genomic changes in the X, S, basal core promoter (BCP), and precore regions of hepatitis B virus (HBV), and evaluate their role in the development of hepatocellular carcinoma (HCC) in chronic hepatitis B (CHB) patients with genotype C HBV. As the results, it was suggested that T1753V mutation in X region might significantly increase the risk of HCC development in CHB patients with genotype C HBV. Also, the BCP mutations might act in synergy with T1753V or G1896A mutation, and with pre-S deletion to promote the development of HCC in these patients.

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INTRODUCTION

Worldwide, hepatocellular carcinoma (HCC) is one of the most common malignant tumors[1]. One of the major risk factors closely associated with HCC development is chronic hepatitis B virus (HBV) infection, which in combination with the hepatitis C virus accounts for more than 80% of HCC worldwide[2]. The specific details of the hepatocarcinogenesis from the initial HBV infection is still unclear, although it has been suggested that many factors including but not limited to sex, age, viral genotype environmental factors, and genetic susceptibility all play an important role in the multistage process of HCC development[3].

Due to the high degree of genetic heterogeneity of the virus, there may be multiple mechanisms by which HBV causes HCC. Some studies have found that HBV can directly provoke hepatocarcinogenesis through chromosomal integration or transactivation of cellular genes[3,4]. Regardless, many of these molecular changes indirectly or directly leading to the development of HCC can be linked to genomic changes within the virus.

Various mutations in the HBV genome have been strongly associated with the development of HCC. A significant amount of evidence points to specific genetic mutations as essential viral factors contributing to the development of HCC that could serve as important prognostic biomarkers of the disease. The substitution of A by T at nucleotide 1762 (A1762T) and of G by A at nucleotide 1764 (G1764A) in the basal core promoter (BCP) region is the most common mutation in the HBV genome. This BCP mutation has been associated with higher occurrence of HCC[5,6]. In addition, it has been reported that other mutations of HBV such as G to A transition at nucleotide 1896 (G1896A) in the precore (PC) region, substitution from C to T at nucleotide 1653 (C1653T) and from T to either C/A/G at nucleotide 1753 (T1753V) in the X region, and the deletion in the pre-S region, may be associated with HCC, although their exact roles are still unknown[7-12].

Moreover, it has been demonstrated that genotype C HBV is strongly associated with mutations in the core promoter region and these particular mutations have been shown to be independent risk factors for HCC development[6,13,14]. Consequently, those infected with genotype C HBV may have a poorer prognosis with more aggressive liver disease[11].

Individual mutations within the HBV genome have been studied in order to prove their role in HCC development in chronic hepatitis B patients. Nevertheless, combined mutations in the HBV genome must also be recognized to have the potential to serve as predictive markers for HCC due to the potential ability of the virus to acquire mutations over time. Although there are few reports about the effects of combined mutations of HBV on HCC development, these results are still controversial.

Therefore, in this study, we intended to investigate the genomic changes in the X, S, BCP, and PC regions of HBV. And also we aimed to evaluate their roles in the development of HCC in patients chronically infected with genotype C HBV.

MATERIALS AND METHODS

Subjects

This study included 240 patients with chronic hepatitis B (CHB) diagnosed at Asan Medical Center, Seoul, Korea, between 1991 and 1998. All patients fulfilled

Table 1 Baseline characteristics of patients with chronic hepatitis B

Variables	*n* = 240
Age, yr[1]	48 (27-86)
Gender, M/F	201/39 (84/16)
[1]Platelet, × 10^3/mm^3	176 (62-556)
Prothrombin time, %[1]	87 (30-147)
ALT, IU/L[1]	149 (10-2170)
Total bilirubin, mg/dL[1]	0.8 (0.3-15.4)
Albumin, g/dL[1]	4.1 (2.2-5.2)
Serum AFP, ng/mL[1]	5.9 (1-2150)
Child-Pugh class, A/B/C	216/15/0 (94/6/0)
HBeAg positivity	195 (81)
Serum HBV DNA, copies/mL[1]	4.2×10^6 (ND-1.1×10^9)
Presence of HBV genotype C	240 (100)
Follow-up periods, mo[1]	105 (1-237)

[1]Median (range). ALT: Alanine aminotransferase; AFP: α-fetoprotein; HBeAg: Hepatitis B e antigen; ND: Not-detected.

the following requirements: (1) seropositivity for hepatitis B surface antigen for at least 6 mo; (2) age > 20 years; and (3) diagnosis with genotype C HBV. The exclusion criteria were as follows: (1) co-infection with hepatitis C virus, hepatitis D virus or human immunodeficiency virus; or (2) diagnosis with HCC at the time of screening.

The patients were followed up regularly at 3-6 month intervals. Serum biochemistry, hematology, HBV DNA titers, hepatitis B e antigen (HBeAg), and anti-HBe, were obtained at each visit. Serum alpha-fetoprotein (AFP), and imaging studies such as ultrasonography or dynamic computed tomographic scan were also performed for the surveillance of HCC development during follow-up. The diagnosis of HCC was determined using AASLD guidelines requiring typical imaging patterns on dynamic CT scan or MRI or pathologic findings[15]. This study was approved by the Institutional Review Board of Asan Medical Center.

HBV genotyping and detection of HBV mutations

HBV DNA was extracted from patient's serum or liver tissue samples using QIAmp DNA extraction kit (Qiagen K.K., Tokyo, Japan). The HBV genotypes were determined by polymerase chain reaction-restriction fragment length polymorphism of the surface gene of the HBV genome. False positives were avoided by preventing cross-contamination and duplicating the tests in order to validate the results.

The sequence was analyzed by amplifying the entire X, S, PC/core, and BCP regions of the HBV genomes. These regions of HBV genome were then sequenced using a direct sequencing method. The methods used for the genotyping and sequencing of the HBV genome were detailed in our previous study[6]. The sequencing conditions, including A1762T or G1764A in the BCP region, G1896A in the PC region, C1653T or T1753V in the region encoding HBx, and the pre-S deletion mutation were specified in the protocol for the Taq DyeDeoxy Terminator Cycle Sequencing Kit (ABI).

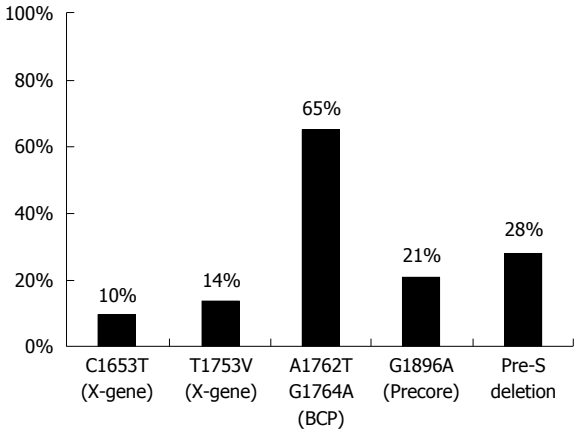

Figure 1 Prevalence of genomic changes in genotype C hepatitis B virus.

Statistical analysis

All variables are expressed as the median (range) or number (percentage). Follow-up duration was calculated from the date of enrollment to the last visit and time to recurrence was defined as the duration from enrollment to HCC diagnosis. To identify factors predisposing the development of HCC, the Kaplan-Meier method and log-rank test were used. And also multivariate analysis was performed using the Cox proportional hazards model. All significance tests were two-tailed, and $P < 0.05$ was considered to be statistically significant. All statistical analyses were performed using the SPSS statistical software package (version 20; SPSS Inc., Chicago, IL).

RESULTS

Baseline characteristics

The demographic data of the 240 patients with CHB are summarized in Table 1. All of the patients had a genotype C HBV infection. About 84% of the patients were male and the median age was 48 years (range, 27-86 years). More than 90% of the patients had relatively well-preserved liver function with Child-Pugh Class A and the median serum alanine aminotransferase (ALT) level was 149 IU/L (range, 10-2170 IU/L). Over 80% of the study subjects were positive for HBeAg. The median follow-up period was 105 mo (range, 1-237 mo).

Prevalence of HBV genomic mutations in CHB patients

The frequencies of genomic mutations in HBV are shown in Figure 1. The C1653T mutation in the X region was found in 10% of the study cases (n = 25). Another mutation in the X region, T1753V, was seen in 14% (n = 33). The BCP double mutation, A1762T/G1764A was detected in 65% (n = 157). The G1896A

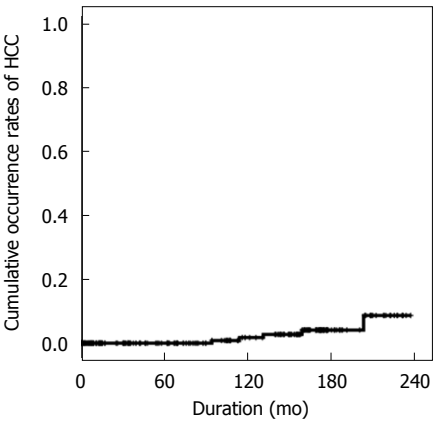

Figure 2 Overall occurrence rates of hepatocellular carcinoma in chronic hepatitis B patients with genotype C hepatitis B virus. HCC: Hepatocellular carcinoma.

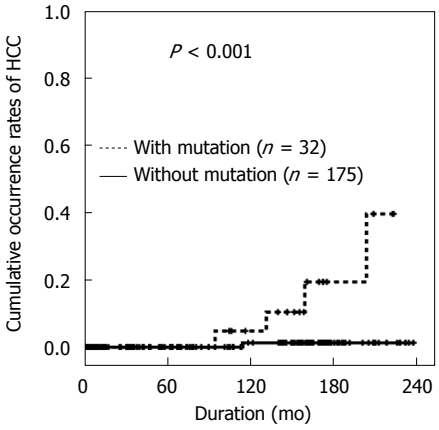

Figure 3 Cumulative occurrence rates of hepatocellular carcinoma in relation to the T1753V mutation in the X region. HCC: Hepatocellular carcinoma.

Table 2 Univariate analysis of risk factors for hepatocellular carcinoma development

Variables	RR (95%CI)	*P* value
Genomic changes		
C1653T in X region	1.583 (0.176-14.210)	NS
T1753V in X region	17.565 (1.956-157.737)	< 0.01
A1762T/G1764A in BCP	30.512 (0.004-230706)	NS
G1896A in PC	5.765 (0.519-64.076)	NS
Pre-S deletion	2.491 (0.502-12.370)	NS
Clinical characteristics		
Age > 50 yr	57.5 (0.035-93428)	NS
Male	27.3 (0.001-547718)	NS
ALT > 80 IU/L	0.55 (0.091-3.29)	NS
PT ⩽ 70 %	4.12 (0.682-24.845)	NS
Serum AFP > 200 ng/mL	0.044 (0.00-209046)	NS
HBeAg positive	0.034 (0.00-360.724)	NS
HBV DNA > 10^5 copies/mL	0.741 (0.123-4.452)	NS

RR: Relative risk; CI: Confidence interval; NS: Not significant; BCP: Basal core promoter; PC: Precore; ALT: Alanine aminotransferase; PT: Prothrombin time; AFP: α-fetoprotein; HBeAg: Hepatitis B e antigen.

mutation in the PC region was seen in 21% (*n* = 50) and the pre-S deletion was found in 28% (*n* = 67).

Overall occurrence rates of HCC in CHB patients

During follow-up, 6 of 240 patients with CHB (3%) newly developed HCC. The 5-, 10-, and 15-year overall cumulative occurrence rates of HCC were 0%, 1.8% and 4.1%, respectively (Figure 2).

Development of HCC in relation to individual genomic mutations in HBV

The presence of the T1753V mutation in the X region was significantly associated with the development of HCC (Figure 3). The 10- and 15-year cumulative occurrence rates of HCC were 5% and 19%, respectively, in patients with the T1753V mutation, which were significantly higher than the 1% 10- and 15-year rates seen in those with wild-type HBV ($P < 0.001$). However, other point mutations, such as C1653T in the X region, the BCP double mutation A1762T/G1764A, G1896A in the PC region, and the pre-S deletion, did not affect HCC occurrence (Table 2).

Effects of combined mutations in HBV genome on HCC development

The occurrence rates of HCC were assessed in relation to the presence of combined mutations to evaluate the synergic effects of genomic changes in HBV. The combination of the BCP double mutations A1762T/G1764A and the T1753V mutation had a close association with HCC development ($P < 0.01$) (Figure 4A). Patients with both BCP A1762T/G1764A mutations and the G1896A mutation also had a significantly higher occurrence rate of HCC ($P < 0.05$) (Figure 4B). In addition, coexistence of BCP double mutations and pre-S deletion showed the higher HCC occurrence rates ($P < 0.05$) (Figure 4C). However, the combination of BCP double mutations and C1653T in the X region was not significantly associated with HCC development (Figure 4D).

Predisposing factors of the development of HCC in patients with CHB

The occurrence rates of HCC in relation to baseline characteristics were also evaluated (Table 2). Age and gender did not showed significant association with the occurrence of HCC in univariate analysis. In addition, other clinical characteristics, such as serum ALT level, prothrombin time, serum AFP level, HBeAg positivity, and HBV DNA titers, did not affect HCC development. In multivariate analysis, the presence of the T1753V mutation in the X region was an independent risk factor for the development of HCC (relative risk 14.03, $P < 0.05$) (Table 3).

DISCUSSION

The impact of genomic changes in HBV on the clinical course of the infection-from the initial viral infection to HCC development-has been of great interest due to the unclear nature of the relationship between

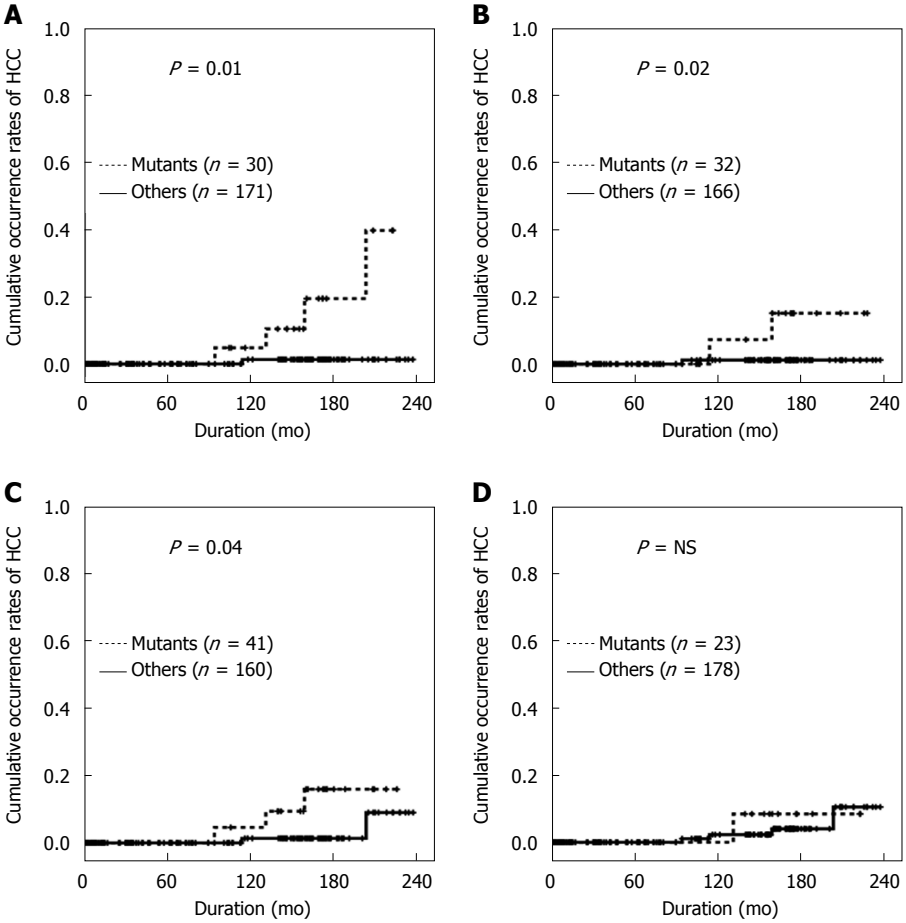

Figure 4 Cumulative occurrence rates of hepatocellular carcinoma in relation to the combination. A: The BCP A1762T/G1764A double mutation and with the T1753V mutation in the X region; B: The BCP A1762T/G1764A double mutation and the G1896A mutation in the PC region; C: The BCP A1762T/G1764A double mutation and the pre-S Deletion; D: The BCP A1762T/G1764A double mutation and the C1653T mutation in the X region. HCC: Hepatocellular carcinoma.

Table 3 Multivariate analysis of predisposing factors for hepatocellular carcinoma development

Variables	RR (95%CI)	*P* value
Age > 50 yr	102903 (0.000-1838)	NS
Male	196796 (0.000-2834)	NS
T1753V in X region	14.029 (1.568-125.550)	< 0.05

NS: Not significant.

the two components. Our work clarifies some of the uncertainty because our results suggest that patients with the T1753V mutation in the X region of the HBV genome are more likely to develop HCC as a result of their chronic HBV infection, especially that of genotype C. The HBx, a nonstructural regulatory protein of HBV, may play an essential role in hepatocarcinogenesis in the context of HBV. Studies of HBx transgenic mice have provided limited results regarding the role of the X-gene in live tumor formation[3,7,11]. Moreover, the exact role of the genomic mutations in the X region is still unclear. However, the present findings indicated that the T1753V mutation in the X region is an independent risk factor for the development of HCC in patients with CHB. Our results thus suggest that an amino acid change at nucleotide 1753 in the X region can affect the function of the HBx protein and thereby contribute to the process of hepatocarcinogenesis[7,11,16]. Therefore, it seems likely that mutations in the X region resulting in the translation of a truncated HBx protein contribute to the initiation of tumor formation in the liver[3,17].

The BCP double mutation A1762T/G1764A is one of the most common mutations in the HBV genome. The current study also showed high prevalence (65%) of A1762T/G1764A in patients with CHB of genotype C. However, the BCP double mutation was not significantly associated with HCC development. Nevertheless, we found that A1762T/G1764A tended to have high occurrence rates of HCC, although the results were not statistically significant. Interestingly, the BCP double mutation A1762T/G1764A significantly contributed to HCC development when it was combined with other individual mutations such as T1753V in the X region, G1896A in the PC region, or the pre-S deletion. Thus, the BCP mutations may also be associated with hepatocarcinogenesis[13,18]. The BCP mutations may change the viral pre-genomic secondary structure or increase the transcription of the pre-genomic RNA, consequently, the BCP mutations

can increase viral replication[14,19].

The PC mutation terminates the translation of HBeAg by creating a premature stop codon in the PC gene[6,7,20]. However, the exact role of this mutation is unclear because, although the mutation has been demonstrated to cause more severe liver disease, it was frequently detected in HBeAg-negative asymptomatic carriers[9,21,22]. In particular, one study has shown that the PC 1896 mutation alone appears to have no direct pathogenic role in HBV genotype C[21]. Our results also showed that the PC G1896A mutation alone was not significantly associated with HCC development, but a combination of the PC mutation and the BCP double mutation significantly influenced HCC occurrence.

Truncated pre-S2/S sequences can often be found in HBV DNA integration sites in HCC[3,23]. As in our present results, a recent study in Taiwan showed that the combination of the pre-S deletion mutation and the BCP double mutation was significantly associated with the development of HCC[6,24]. It is possible that hepatocytes expressing altered large and middle surface proteins encoded by the mutated S gene have a potential growth advantage[10,25]. Moreover, the pre-S mutant has been found to upregulate cyclin A expression and induce nodular proliferation of hepatocytes[10,17]. The modified surface antigen may induce oxidative DNA damage and mutations in hepatocytes in the late stages of a chronic HBV infection[10,26].

One limitation of our present study was the small number of newly developed HCC cases during follow-up. Out of 240 patients with CHB, only 6 patients developed HCC. However, most subjects had CHB, not liver cirrhosis. In addition, the median follow-up duration was about 9 years, which may be insufficient duration for HCC development, especially in patients with CHB. Nevertheless, our current study is one of the largest of its kind to date and had a longer follow-up period than other similar reports.

In conclusion, the presence of the T1753V mutation in the X region significantly increases the risk of HCC development in patients with genotype C CHB. The BCP mutations may act in synergy with the T1753V mutation in the X region, G1896A in the PC region, and with the pre-S deletion to significantly increase the occurrence of HCC in CHB patients with genotype C HBV.

COMMENTS

Background

Hepatitis B virus (HBV) is the most common risk factor for hepatocellular carcinoma development. Especially, among the various characteristics of HBV, genomic variation of the virus may effect on hepatocellular carcinoma (HCC) development directly. However, the exact role of the genomic mutation of HBV is still controversial for hepatocarcinogenesis. Thus, it is very important to clarify obvious function of the genomic mutation for the development of HCC in patients with HBV infection.

Research frontiers

The authors are part of the leading group in the studies of carcinogenesis for HBV associated HCC. The authors believe that some mutations in the basal core promoter, the precore, and the X regions may be associated with HCC development. We provide reasonable evidence to support the hypothesis which we have made from this paper.

Innovations and breakthroughs

Generally agreed by the most researchers on genomic changes of HBV are able to effect on the development of HCC. However, their opinions are far from being unanimous as to obvious role of each mutation for the development of HCC. This paper shows that the T1753V mutation in X region plays an important role as the risk factor for HCC development. Moreover, combined mutations of the basal core promoter mutation and other mutations such as T1753V, G1896A, and the pre-S deletion are also the risk factors for the development of HCC in patients with genotype C HBV infection.

Applications

High risk group for HCC development can be identified among the patients infected with genotype C HBV. Furthermore, the authors can establish efficient process to detect HCC earlier and also the authors may prevent the development of HCC ultimately.

Terminology

HBV is a member of hepadnaviridae family, and it can cause acute hepatitis, fulminant hepatitis, asymptomatic carrier state, chronic hepatitis, cirrhosis, and HCC. Especially, HBV is the most common risk factor for HCC development. Because HBV has the high degree of genetic diversity, it is considered that the genomic changes of HBV can influence on the carcinogenesis of HCC, although the exact role of each genomic mutation is not obvious. HCC is a primary cancer of the liver and usually occurs in patients with chronic liver disease, especially chronic hepatitis B. Multiple mechanisms impact on the development of HCC and some of mechanisms has not been fully verified as yet.

Peer-review

Excellent study has been performed by Danbi Lee *et al* reporting about the association between genomic changes of genotype C HBV and HCC development. This study definitely help to verify mechanism of HCC development. Congratulation to authors for the valuable results for role of genetic mutation of HBV for the hepatocarcinogenesis.

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ORIGINAL ARTICLE

Retrospective Study

Last line therapy with sorafenib in colorectal cancer: A retrospective analysis

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Abstract

AIM: To analyze the efficacy of last line sorafenib treatment in colorectal cancer patients.

METHODS: All patients receiving chemotherapy for colorectal cancer in the outpatient clinic of the University of Mainz since 2006 were retrospectively analyzed for last line sorafenib exposure. Charts of identified patients were analyzed for clinic-pathological parameters, like data on gender, age, date of initial diagnosis, UICC stage, number and kind of the pre-therapies, therapy start and end of sorafenib, sorafenib mediated treatment cessation, side effects, response rates, time to progression and overall survival.

RESULTS: Ten patients with a median of 3.0 prior chemotherapy lines had received a last line sorafenib therapy either alone (10%) or in combination with

5-fluorouracil derivates (90%). All patients suffered from colorectal cancer stage UICC 4 and were routinely seen in 2-wk intervals in the oncology outpatient clinic. Median duration of treatment was 142.0 d. At 8 wk 80% of patients showed stable disease but we did not observe any remissions. Median time to progression was 140.5 d (4.7 mo), while median overall survival reached 176.5 d. One patient ceased treatment due to side effects. Reason for treatment stop was bleeding complication in one case and non-specified sorafenib intolerance in another case. Due to the retrospective approach we did not further quantify side effects.

CONCLUSION: This retrospective analysis encourages further investigation of sorafenib in colorectal cancer last line therapy.

Key words: Colorectal cancer; Sorafenib; Regorafenib; Chemotherapy; Last line

Core tip: In this restrospective analysis we demonstrate that sorafenib monotherapy or in combination with 5-fluorouracil derivates seems to be feasible. Eighty percent of the patients showed stable disease with a median time to progression of 140.5 d and acceptable toxicity profile. In our eyes, the reported overall as well as progression free survival under sorafenib treatment are of clinical and financial interest.

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INTRODUCTION

Colorectal cancer ranges among the most frequent malignancies in Western countries[1,2]. Annually, more than 1.2 million patients are diagnosed with colorectal cancer resulting in more than 600000 death each year (1 Sora). Survival is delineated by local recurrence and tumor dissemination[3]. Noteworthy, 50% of patients develop metastases in the due course of the disease (2 Sora).

Due to improved therapeutic strategies, the overall survival in metastatic stage Ⅳ colorectal cancer has increased from eight months to more than two years during the last decade. Chemotherapeutics, such as platinum derivates (oxaliplatin) or topoisomerase Ⅱ inhibitors (irinotecan) as well as the introduction of biologicals targeting tumor neo-vascularisation (anti-VEGF: bevacizumab and aflibercept) or growth-signaling (anti-EGFR: cetuximab and panitumumab) have significantly augmented response rates and prognosic parameters[4-10].

Those new strategies have resulted in an unexpected dilemma: Numerous patients in good condition have experienced progression following treatment with all available agents. This therapeutic gap has recently been targeted by the multi-tyrosine kinase inhibitor regorafenib[11]. Last line treatment with regorafenib resulted in a progression free survival of 1.9 mo (*vs* 1.7 mo in the placebo arm) and an overall survival of 6.4 mo (*vs* 5.0 mo in the placebo arm). Receptor tyrosine kinases (RTKs) are transmembrane-receptors containing extracellular ligand-binding domains connected to intracellular catalytic domains[12]. The growth factors VEGF/PDGF/EGF and their receptors VEGFR1-3, PDGFRα/β and EGFR are critical in the process of (lymphatic) neo-angiogenesis and dissemination in human cancer[13-17].

Inhibition of RTKs with sorafenib has been successful in renal and hepatocellular cancer[18,19]. Two phase I studies revealed a disease stabilization in pretreated colorectal cancer patients receiving sorafenib in combination with either irinotecan or oxaliplatin[20,21]. Therefore, the impact of combinational therapies (sorafenib + chemotherapy) remains controversial.

However due to a lack of treatment options in the augmenting number of colorectal cancer patients pretreated with all available chemotherapeutic and biological options, we identified 10 patients which had received off-label sorafenib within a risk sharing program of Bayer Healthcare. The current publication reports on the results of those patients in a retrospective approach.

MATERIALS AND METHODS

Analyses

We retrospectively analyzed all medical records of colorectal cancer patients which received any treatment in the outpatient clinic of the university if Mainz between January 1, 2007 and December 31, 2011 in order to identify patients that had received sorafenib as last line treatment. We then retrospectively collected and analyzed data from the medical records. In particular we collected data on gender, age, date of initial diagnosis, UICC stage, number and kind of the pre-therapies, therapy start and end of sorafenib, sorafenib mediated treatment cessation, progression free survival (PFS), overall survival (OS) and relative risk.

RESULTS

Patient characteristics

We identified 10 patients, which had received off-lable sorafenib after entering a risk sharing program of Bayer Healthcare. All patients were routinely seen

Table 1 Patient characteristics

Patient characteristics	*n* (%)
Total number	10.0
Average age (yr)	65.4
Gender	
Female	2 (20)
Male	8 (80)
Location	
Colon	8 (80)
Rectum	2 (20)
UICC	
1 + 2 + 3	0 (0)
4	10 (100)
Metastases	
Liver	6 (60)
Lung	8 (80)
Lymph nodes	5 (50)
Prior ctx regimen	
3	7 (70)
4	2 (20)
5	0 (0)
6	1 (10)

in 2-wk intervals in the oncology outpatient clinic. At visits following assessments were done: general condition of the patients, blood counts, side effects. Staging analyses (CT scan abdomen and thorax) were done every 8 wk. All patients (100%) suffered from colorectal cancer stage UICC 4. Eighty percent of patients were male, while 20% were of female gender. Average patient age was 65 years. One hundred percent of patients were in good condition as indicated by an ECOG of 0-1. Patients had received an average of 3 prior chemotherapy regimens prior to treatment with sorafenib (Tables 1 and 2).

Treatment characteristics

One patient (10%) had received a sorafenib monotherapy, while 9 patients (90%) had had a combination of sorafenib with different 5-fluorouracil (5-FU) derivates. 5-FU derivates applied were intravenous 5-FU (*n* = 2; folinic acid 400 mg/qm d1, 5-FU 400 mg/q.m. bolus d1, 2400 mg/q.m. d1 and d2), capecitabine (*n* = 4; dose 2000 mg/q.m. d1-d14) or Tegafur-Uracil (*n* = 3; 300 mg/q.m. and Calciumfolinat 90 mg/d d1-d28). All patients were initially administered a reduced dose of sorafenib of 400 mg/d (200 mg b.i.d.). The dose was adjusted to 800 mg/d (400 mg b.i.d.) after 1 to 2 wk. Treatment duration was 142 d in median. Maximal treatment duration was 176 d. Eighty percent of patients (8/10) received treatment until progression, while 20% (2/10) ceased treatment due to side effects after average treatment duration of 56 d. Reason for treatment stop was bleeding complication in one case and non-specified sorafenib intolerance in another case. Due to the retrospective approach we did not further quantify side effects (Table 3).

Survival and response parameters

At the first staging at treatment week 6, 80% of patients (8/10) revealed stable disease (SD) as compared to progressive disease (PD) in 20% (2/10; one of both patients had died after 21 d of treatment due to clinical progress). No partial or complete responses were observed. At week 12 only six patients were evaluable. Of those 50% (3/6) revealed stable disease and 50% (3/6) progressive disease. Median PFS was 140 d, median OS was 176 d (Figures 1 and 2).

Adverse events

The most frequent sorafenib-related adverse events of grade 3 or higher were fatigue (30%), anemia (20%), emesis (10%), mucositis (10%), pain (10%), leucocytopenia (10%) and thrombocytopenia (10%; Table 4). Relevant sorafenib-related adverse events grade 1 or 2 were fatigue (70%), diarrhea (40%), anemia (40%), mucositis (30%), hand foot syndrome (30%) and thrombocytopenia (30%) among others (Table 4).

DISCUSSION

As to our best knowledge, this is the first retrospective study investigating last line sorafenib in colorectal cancer patients. Prospective studies have not been performed or published, so far.

Due to augmented therapeutic options, the overall survival in metastatic stage Ⅳ colorectal cancer has increased from eight months to more than two years during the last decade[4-7]. Those new strategies have resulted in an unexpected dilemma: Numerous patients in good condition have experienced progression following treatment with all available agents. This therapeutic gap has recently been targeted by the multi-tyrosine kinase inhibitor regorafenib[11]. Last line treatment with regorafenib resulted in a progression free survival of 1.9 mo (*vs* 1.7 mo in the placebo arm) and an overall survival of 6.4 mo (*vs* 5.0 mo in the placebo arm). In the light of the regorafenib approval in 2013 we decided to analyze the efficacy of sorafenib in a retrospective approach. Diverse colorectal cancer patients in good condition had previously received sorafenib in a last line approach over the recent years prior to admission of regorafenib.

Our retrospective data must be handled with care due to their limitation by the retrospective approach and the small number of patients. All patients were UICC stage Ⅳ, in good condition and had received an average of 3 prior chemotherapy regimens. However, the majority of patients were of male gender, thus not representing the normal distribution of colorectal cancer among genders.

Tolerability of last line sorafenib was generally good: 30% of patients developed a grade 3/4 fatigue, 20% a grade 3/4 anemia and respectively 10% a grade 3/4 emesis, mucositis, leucocytopenia or thrombocytopenia. Thus grade 3/4 fatigue, anemia and thrombocytopenia seems to occur more often than

Table 2 Detailed description of patients at therapy start

Patient	Age	Gender	Primary	UICC	Liver metastases	Lunge metastases	Number of pre-therapies
1	68	M	Sigma	IV	1	1	3
2	52	M	Colon desc.	IV	0	1	3
3	51	M	Sigma	IV	1	1	3
4	81	M	Rectum	IV	1	1	3
5	59	F	Colon desc.	IV	1	1	3
6	74	M	Sigma	IV	0	1	3
7	74	M	Rectum	IV	1	0	4
8	65	F	Sigma	IV	1	1	6
9	69	M	Sigma	IV	0	1	3
10	61	M	Colon desc.	IV	0	0	4

Table 3 Detailed treatment description of patients

Pt.	Comb. partner	6-wk		12-wk		PFS (d)	OS (d)
		Response	% change	Response	% change		
1	5-FU	SD	-13%	SD	-7%	144	165
2	None	0	0	0	0	21	21
3	TU	SD	12%	0	0	79[1]	163
4	5-FU	PD	35%	0	0	41	382
5	C	SD	-6%	PD	32%	149	302
6	TU	SD	-2%	0	0	32[2]	113
7	C	SD	-21%	SD	5%	137	188
8	C	SD	-4%	PD	24%	199	296
9	C	SD	3%	PD	32%	177	147
10	TU	SD	5%	SD	6%	188	447

[1]Treatment stop due to bleeding complication; [2]Treatment stop due to sorafenib allergy. TU: Tegafur Uracil; C: Capecitabine; SD: Stable disease; PD: Progressive disease; PFS: Progression free survival.

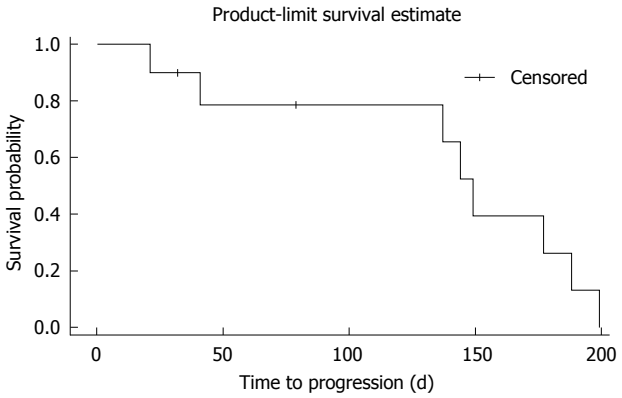

Figure 1 Progression free survival. Kaplan-Meier analysis, retrospective population. Median progression free survival 140.5 d.

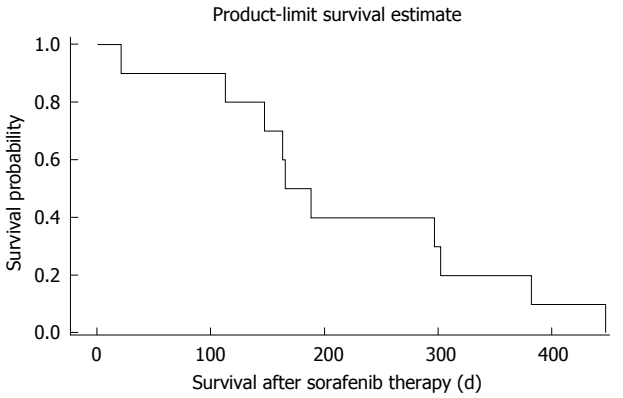

Figure 2 Overall survival. Kaplan-Meier analysis, retrospective population. Median progression free survival 176.5 d.

under regorafenib (10%, 3% and 4%, respectively)[11]. However, due to the low number of cases this data is at best descriptive.

We were surprised to find a median PFS of 4.7 mo (140 d) among our patients as compared to 1.9 mo under regorafenib treatment as reported by Axel Grothey and coauthors. In addition our retrospective analysis revealed an overall survival of 5.9 mo (176 d) and thus is in line with the reported overall survival of last line regorafenib (6.4 mo *vs* 5.0 mo in the placebo arm). As to be expected we did not observe any remissions but found an interesting effect in disease stabilization (80% at week 8). Noteworthy, the majority of patients (90%) had received a combination of sorafenib with 5-FU (or its pro-drugs) and only one patient had ceased treatment due to side effects.

However, the combination of sorafenib with 5-FU might not explain the observed effects. As reported by our own group, we previously studied the combination of sorafenib with 5-FU *in vitro* as well as *in vivo* in xenograft models[22]. We demonstrated that a sorafenib-monotherapy (5 mg/kg; approximately 400 mg in an 80 kg patient) was equally effective as a combination therapy of both sorafenib and 5-FU. Thus,

Table 4 Adverse events (CTC AE3.0)

Patient	Ⅰ°-Ⅱ°	Ⅲ°-Ⅳ°	Total
Fatigue	70% (7/10)	30% (3/10)	100%
Nausea	20% (2/10)	0% (0/10)	20%
Emesis	20% (2/10)	10% (1/10)	30%
Diarrhea	40% (4/10)	0% (0/10)	40%
Mukositis	30% (3/10)	10% (1/10)	40%
Rash	20% (2/10)	0% (0/10)	20%
HFS	30% (3/10)	0% (0/10)	30%
PNP	20% (2/10)	0% (0/10)	20%
Pain	20% (2/10)	10% (1/10)	30%
Weight loss	10% (1/10)	0% (0/10)	10%
Infection	20% (2/10)	0% (0/10)	20%
Anemia	40% (4/10)	20% (2/10)	60%
Thrombopenia	30% (3/10)	10% (1/10)	40%
Leucopenia	20% (2/10)	10% (1/10)	30%

a combination therapy did not result in any additive effects, but might add adverse events. However, these data became available only after the off-label treatment of colorectal cancer patients.

In our eyes, the reported overall as well as progression free survival under sorafenib treatment are of clinical and financial interest, as treatment costs of regorafenib sum up to €5573 per 28-d-cycle as compared to €2611 for its predecessor sorafenib. Therefore a trial focusing on non-inferiority for sorafenib *vs* regorafenib might be feasible.

COMMENTS

Background

Improved therapeutic strategies have resulted in an unexpected dilemma: Numerous patients in good condition have experienced progression following treatment with all available agents. This therapeutic gap has recently been targeted by the multi-tyrosine kinase inhibitor regorafenib. Last line treatment with regorafenib resulted in a progression free survival of 1.9 mo (*vs* 1.7 mo in the placebo arm) and an overall survival of 6.4 mo (*vs* 5.0 mo in the placebo arm). Receptor tyrosine kinases (RTKs) are transmembrane-receptors containing extracellular ligand-binding domains connected to intracellular catalytic domains. The growth factors VEGF/PDGF/EGF and their receptors VEGFR1-3, PDGFRα/β and EGFR are critical in the process of (lymphatic) neo-angiogenesis and dissemination in human cancer. Inhibition of RTKs with sorafenib has been successful in renal and hepatocellular cancer. Two phase I studies revealed a disease stabilization in pretreated colorectal cancer patients receiving sorafenib in combination with either irinotecan or oxaliplatin. Therefore, the impact of combinational therapies (sorafenib + chemotherapy) remains controversial.

Research frontiers

Due to novel therapeutic approaches patients with stage IV colorectal cancer show an improvement in overall survival. In this palliative setting it remains a major goal to reduce therapy induced toxicity and still preserve improvement of progression free survival or overall survival.

Innovations and breakthroughs

Ten patients with a median of 3.0 prior chemotherapy lines had received a last line sorafenib therapy either alone (10%) or in combination with 5-fluorouracil derivates (90%). All patients suffered from colorectal cancer stage UICC 4 and were routinely seen in 2-wk intervals in the oncology outpatient clinic. Median duration of treatment was 142.0 d. At 8 wk 80% of patients showed stable disease. Therefore sorafenib could be a very efficient and cost effective therapeutic last line approach to compared to regorafenib.

Applications

The authors evaluate a cost effective last line therapeutic approach for colorectal cancer patient with a favorable toxicity profile.

Terminology

Receptor tyrosine kinases (RTKs), are transmembrane-receptors containing extracellular ligand-binding domains connected to intracellular catalytic domains. The growth factors VEGF/PDGF/EGF and their receptors VEGFR1-3, PDGFRα/β and EGFR are critical in the process of (lymphatic) neo-angiogenesis and dissemination in human cancer. Sorafenib and regorafenib are inhibitors for these RTKs.

Peer-review

The retrospective study in order to analyze the efficacy of sorafenib treatment in colorectal cancer present a small number of patients, but the results are valuable.

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Retrospective Study

Association of HER2 status with prognosis in gastric cancer patients undergoing R0 resection: A large-scale multicenter study in China

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Abstract

AIM: To determine whether the positive status of human epidermal growth receptor 2 (HER2) can be regarded as an effective prognostic factor for patients with gastric cancer (GC) undergoing R0 resection.

METHODS: A total of 1562 GC patients treated by R0 resection were recruited. HER2 status was evaluated in surgically resected samples of all the

patients using immunohistochemical (IHC) staining. Correlations between HER2 status and clinicopathological characteristics were retrospective analyzed. Hazard ratios (HRs) and 95% confidence intervals (CIs) were estimated using Cox proportional hazard model, stratified by age, gender, tumor location and tumor-node-metastasis (TNM) stage, with additional adjustment for potential prognostic factors.

RESULTS: Among 1562 patients, 548 (positive rate = 35.08%, 95%CI: 32.72%-37.45%) were HER2 positive. Positive status of HER2 was significantly correlated with gender (P = 0.004), minority (P < 0.001), tumor location (P = 0.001), pathological grade (P < 0.001), TNM stage (P < 0.001) and adjuvant radiotherapy (74.67% *vs* 23.53%, P = 0.011). No significant associations were observed between HER2 status and disease free survival (HR = 0.19, 95%CI: 0.96-1.46, P = 0.105) or overall survival (HR = 1.19, 95%CI: 0.96-1.48, P = 0.118) using multivariate analysis, although stratified analyses showed marginally statistically significant associations both in disease free survival and overall survival, especially among patients aged < 60 years or with early TNM stages (Ⅰ and Ⅱ). Categorical age, TNM stage, neural invasion, and adjuvant chemotherapy were, as expected, independent prognostic factors for both disease free survival and overall survival.

CONCLUSION: The positive status of HER2 based on IHC staining was not related to the survival in patients with GC among the Chinese population.

Key words: Human epidermal growth receptor 2; Gastric cancer; R0 resection; Chinese population; Prognostic factors

Core tip: The study retrospectively analyzed the associations between the positive status of human epidermal growth receptor (HER) 2 and survival of patients with gastric cancer (GC) undergoing R0 resection among the Chinese population, and found that the positive status of HER2 based on immunohistochemical staining was not related to survival in GC patients.

Shen GS, Zhao JD, Zhao JH, Ma XF, Du F, Kan J, Ji FX, Ma F, Zheng FC, Wang ZY, Xu BH. Association of HER2 status with prognosis in gastric cancer patients undergoing R0 resection: A large-scale multicenter study in China. *World J Gastroenterol* 2016; 22(23): 5406-5414 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i23/5406.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i23.5406

INTRODUCTION

The human epidermal growth receptor 2 (HER2) is now well recognized as a key factor in the development of certain solid human tumors[1], most notably in breast cancer[2]. It activates numerous downstream pathways in response to extracellular ligands, regulating diverse processes that include differentiation, migration, proliferation and survival[3,4]. Over the last few years, HER2 has been the most frequently studied molecular biological prognostic factors in various tumors[5-7].

In gastric cancer (GC), the ToGA trial was the first study showing the clinical benefit of trastuzumab in combination with chemotherapy for HER2 positive advanced GC patients[8,9]. That is to say, HER2 status is thought to be an important prognostic factor and biologic agent[10-12]. However, a post-hoc subgroup analyses failed to show survival benefits of trastuzumab therapy in Asian populations[8]. Although several studies have evaluated the poor prognosis of GC with HER2 overexpression recently[13,14], the clinical significance of such association remains controversial[14-16]. Therefore, we conducted this retrospective study to examine whether the positive status of HER2 can be regarded as an effective prognostic factor for patients with GC undergoing R0 resection among the Chinese population.

MATERIALS AND METHODS

Ethical statement

All procedures performed in the study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was also approved by institutional review board of Qinghai University Affiliated Hospital and National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College. Additional informed consent was obtained from all individual participants for whom identifying is included in this article.

Patients

In this retrospective study, 1562 patients with GC who underwent R0 resection between December 2009 and December 2011 at Qinghai University Affiliated Hospital, Xining, or National Cancer Center/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, or People's Hospital of Qinghai province, Xining, were recruited. Patients were treated exclusively by total or subtotal gastrectomy with lymphadenectomy according to tumor location; neoadjuvant chemotherapy or radiotherapy was not administered to any patients. Patients' characteristics regarding age (categorical age: <60 years *vs* ⩾ 60 years), gender, minority, family history, tumor location, histological grade, tumor stage, tumor embolus, neural invasion and adjuvant chemotherapy and radiotherapy are listed in Table 1. Each cases was staged according to the tumor-node-metastasis (TNM) cancer staging system of malignant tumors 7th edition advocated by the American Joint Committee on Cancer (AJCC)[17].

Table 1 Baseline and clinicopathological characteristics among gastric cancer patients with different human epidermal growth receptor 2 status *n* (%)

Characteristic	HER2 status		Total	χ^2	*P* value[1]
	Negative (-) *n* = 1014	Positive (+) *n* = 548			
Age					
Median (range)	60 (21-82)	60 (20-84)	59 (20-84)		
< 60 yr	504 (63.72)	287 (36.28)	791 (50.64)	1.012	0.314
⩾ 60 yr	510 (66.15)	261 (33.85)	771 (49.36)		
Gender				8.288	0.004
Female	274 (70.98)	112 (29.02)	386 (24.71)		
Male	740 (62.93)	436 (37.07)	1176 (75.29)		
Minority				11.518	< 0.001
Han population	989 (65.71)	516 (34.29)	1505 (96.35)		
Others	25 (43.86)	32 (56.14)	57 (3.65)		
History of familial cancer				0.144	0.705
No	890 (76.99)	266 (23.01)	1156 (88.64)		
Yes	123 (78.34)	34 (21.66)	157 (11.96)		
Tumor location				10.357	0.001
Non-cardia	624 (62.03)	382 (37.97)	1006 (64.40)		
Cardia	390 (70.14)	166 (29.86)	556 (35.60)		
Histological grade				37.822	< 0.001
High differentiation	31 (40.79)	45 (59.21)	76 (4.87)		
Moderate differentiation	203 (61.89)	125 (38.11)	328 (21.00)		
Low differentiation or Signet-ring cell cancer	762 (68.40)	352 (31.60)	1114 (71.32)		
Early and unreported	18 (40.91)	26 (59.09)	44 (2.82)		
TNM stage				21.640	< 0.001
Ⅰ	207 (55.20)	168 (44.80)	375 (24.01)		
Ⅱ	215 (65.55)	113 (34.45)	328 (21.00)		
Ⅲ	592 (68.92)	267 (31.08)	859 (54.99)		
Tumor embolus				0.034	0.954
No	761 (64.88)	412 (35.12)	1173 (75.10)		
Yes	253 (65.04)	136 (34.96)	389 (24.90)		
Neural invasion				3.054	0.081
No	877 (65.79)	456 (34.21)	1333 (85.34)		
Yes	137 (59.83)	92 (40.17)	229 (14.66)		
Adjuvant chemotherapy				2.872	0.090
No	423 (62.57)	253 (37.43)	676 (43.28)		
Yes	591 (66.70)	295 (33.30)	886 (56.72)		
Adjuvant radiotherapy				6.396	0.011
No	936 (64.11)	524 (35.89)	1460 (93.47)		
Yes	78 (76.47)	24 (23.53)	102 (6.53)		

[1]The parametric *P* value is calculated by t-test for numerical covariates and χ^2 test for categorical covariates. Number of observations in the original data set = 1562. Number of observations used = 1313, owing to the missing value occurring in family history of cancer (249). HER2: Human epidermal growth receptor 2.

All patients were evaluated for disease recurrence and survival status by clinical examinations, upper gastrointestinal endoscopic assessment, and diagnostic imaging (chest radiograph, ultrasonography, computed tomography, or magnetic resonance imaging) every 3 mo during the 1st year and once 6 mo thereafter until death or the last time of follow-up.

Tissue processing

Samples were removed from tumors; grossly necrotic tissue was avoided. Immediately after surgical resection, samples were processed for pathological examination while the remainder was washed with a cold saline solution, divided into aliquots, rapidly transported on ice to the laboratory, and stored at -70 ℃ pending biochemical studies.

Specimens from neoplastic tissues were processed at the same time. They were fixed in buffered 10% formalin, embedded in paraffin, and 4-μm thick sections were cut from the paraffin block of each specimen and applied to slides for immunohistochemical (IHC) staining.

IHC staining and HER2 status

The IHC analysis with the Herecp test was performed according to the manufacturer's instructions. In brief, heat-induced epitope retrieval was performed on the deparaffinized sections in advance by immersing the slides in Epitope Retrieval Solution (10 mm citrate buffer; pH = 6.0), which had been preheated to 95 ℃. They were then placed in a water bath at 95 ℃ for 40 min, followed by incubation for 20 min at room temperature, then endogenous peroxidase was quenched with Peroxidase Blocking Reagent. Next, the slides were incubated at room temperature for 30 min with a ready-to-use rabbit polyclonal antibody to HER,

and the primary antibody was detected by incubation at room temperature for 30 min with Visualization Reagent (dextran polymer conjugated with horseradish peroxidase and goat anti-rabbit immunoglobulin). After washing, slides were developed with Substrate Chromogenic Solution at room temperature for 10 min. The results were scored following the HER2 scoring scheme (scores 0, 1+, 2+ and 3+) in accordance with DFA-approved system for breast cancer[18] and interpreted by two independent pathologists who were blinded to the clinical information, and the inconsistent results were also judged by the third pathologist. Positivity status of HER2 was defined as IHC 3+, and the others were considered as negative.

Statistical analyses

Pearson χ^2 tests or Fisher exact tests were used to explore the correlation between HER2 status and clinicopathological characteristics. Kaplan-Meier method was performed to calculate the disease free survival (DFS) rate and overall survival (OS) rate, and survival curves were compared using log-rank test. Hazard ratios (HRs) and 95% confidence intervals (CIs) were estimated using Cox proportional hazard regression model with and without additional adjustment for potential prognostic factors. The proportional hazard assumption was examined using models that allowed time-dependent HRs combined with curve of log[-log (t)].

All *P*-values were two-sided and *P*-values less than 0.05 were considered statistically significant. All analyses were conducted using SAS version 9.1.3 service pack 4 (SAS Institute, Inc., Cary, NC).

RESULTS

Patients and baseline characteristics

A total of 1562 patients with a median age of 59 years (range: 20-84 years) were recruited and screened for HER2 status by IHC staining, of whom 548 were considered HER2 positive. Baseline and clinical pathological characteristics are outlined in Table 1. Of these 1562 patients, 1176 (75.29%) were male, 1505 (96.35%) were Chinese Han, 157 (11.96%) were reported with a positive history of familial cancer, and 1006 (64.40%) with primary tumor located in gastric were verified by pathology. All the patients were treated by R0 resection, with 389 (24.90%) patients developing tumor embolus. Therefore, all patients in this study had enough tumor samples to conduct the IHC analysis. Poorly differentiated tumors were observed in 71.32% of patients, and 54.99% of patients had pTNM stage Ⅲ disease. Besides, 886 (56.72%) and 102 (6.53%) patients accepted the adjuvant chemotherapy and radiotherapy, respectively, after the surgical treatment owing to the neural invasion (229, 14.66%) or other reasons.

HER2 status and clinicopathological characteristics

Table 1 shows the HER2 status and clinicopathological characteristics between HER2 positive and negative patients. Of all the patients, 548 tested positive for HER2, with a positive rate of 35.08% (95%CI: 32.72-37.45%). No significant differences were observed for categorical age (<60 years *vs* ⩾ 60 years), familial cancer, tumor embolus, neural invasion or adjuvant chemotherapy. We found that the following factors were statistically associated with the HER2 status. Compared with negative status of HER2, patients with positive status had a significantly lower proportion of male gender (62.93% *vs* 37.07%, $P = 0.004$), Chinese Han population (65.71% *vs* 34.29%, $P < 0.001$), GC (62.03% *vs* 37.97%, $P = 0.001$), pTNM stages (Ⅰ: 55.20% *vs* 44.80%, Ⅱ: 65.55% *vs* 34.45% and Ⅲ: 68.92% *vs* 31.08%, respectively, $P < 0.001$) and adjuvant radiotherapy (74.67% *vs* 23.53%, $P = 0.011$). In contrast, compared with patients with positive status of HER2, patients with negative status had a lower proportion of high differentiation (40.79% *vs* 59.21%), whereas a higher proportion of moderate differentiation (38.11% *vs* 61.89%) and low differentiation or signet-ring cell cancer (31.63% *vs* 68.37%) with *P*-values less than 0.001.

HER2 status and DFS

Because of the potential differences in DFS among the patients with negative or positive status of HER2, we estimated the HRs and adjusted HRs. There were no statistically significant associations between the status of HER2 and DFS in GC, regardless of additional adjustment of potential prognostic factors (Table 2).

In stratified analyses, we observed that the risk of disease progression was marginally significantly increased for GC patients in both younger population and older population (⩾ 60 years), although the HR reached statistical significance only in the older group (HR = 1.32, 95%CI: 1.02-1.71, $P = 0.032$). Results were also marginally significant for risk of disease progression in pTNM stage, especially in stage Ⅰ (HR = 1.86, 95%CI: 0.99-3.50, $P = 0.056$) and stage Ⅱ (HR = 1.55, 95%CI: 0.94-2.55, $P = 0.089$) according to the AJCC-TNM Cancer Staging 7th edition (Tables 2 and 3, and Figure 1).

HER2 status and OS

No significant association was observed between HER2 status and OS rate. Further stratified analyses showed marginally statistically significant associations in both the older patients (HR = 1.28, 95%CI: 0.98-1.68, $P = 0.070$) and patients with early pTNM stage (Ⅱ *vs* Ⅰ: HR = 2.03, 95%CI: 0.97-4.23, $P = 0.061$; Ⅲ *vs* Ⅰ: HR = 1.75, 95%CI: 1.04-2.94, $P = 0.035$) (Tables 2 and 3, and Figure 2).

Other prognostic factors

As expected, the estimated parameters of categorical

Table 2 Hazard ratios and 95% confidence intervals for the association between potential prognosis factors and gastric cancer

Characteristic	*n*	DFS				OS			
		HR (95%CI)[1]	*P* value[1]	HR (95%CI)[2]	*P* value[2]	HR (95%CI)[1]	*P* value[1]	HR (95%CI)[2]	*P* value[2]
Age			< 0.001		< 0.001		< 0.001		< 0.001
< 60 yr	792	1		1		1		1	
⩾ 60 yr	772	1.58 (1.35-1.85)		1.42 (1.18-1.71)		1.60 (1.35-1.89)		1.39 (1.15-1.70)	
Gender			0.674		0.992		0.976		0.651
Female	387	1		1		1		1	
Male	1177	0.96 (0.80-1.15)		1.00 (0.82-1.23)		1.00 (0.82-1.21)		1.05 (0.85-1.30)	
Minority			0.364		0.600		0.563		0.797
Han population	1507	1		1		1		1	
Others	57	0.81 (0.51-1.28)		0.87 (0.52-1.46)		0.87 (0.54-1.40)		0.93 (0.55-1.59)	
History of familial cancer			0.367		0.295		0.610		0.523
No	1157	1		1		1		1	
Yes	157	1.12 (0.87-1.44)		1.15 (0.89-1.48)		1.07 (0.82-1.41)		1.09 (0.83-1.44)	
Tumor location			< 0.001		0.743		0.003		0.545
Cardia	1007	1		1		1		1	
Non-cardia	557	1.34 (1.14-1.57)		0.97 (0.81-1.16)		1.29 (1.09-1.53)		0.94 (0.78-1.14)	
TNM stage									
Ⅰ	376	1				1		1	
Ⅱ	329	2.71 (1.92-3.83)	< 0.001	2.61 (1.76-3.87)	< 0.001	3.27 (2.21-4.83)	< 0.001	3.33 (2.14-5.18)	< 0.001
Ⅲ	859	6.25 (4.64-8.44)	< 0.001	6.50 (4.57-9.23)	< 0.001	7.58 (5.37-10.70)	< 0.001	8.70 (5.83-12.98)	< 0.001
Tumor embolus			< 0.001		0.339		< 0.001		0.802
No	1175	1		1		1		1	
Yes	289	1.81 (1.53-2.13)		1.10 (0.90-1.34)		1.70 (1.43-2.03)		0.97 (0.79-1.20)	
Neural invasion			< 0.001		< 0.001		< 0.001		< 0.001
No	1334	1		1		1		1	
Yes	230	1.90 (1.57-2.30)		1.51 (1.21-1.89)		2.06 (1.69-2.52)		1.68 (1.33-2.12)	
Adjuvant chemotherapy			0.008		0.015[1]		0.052		0.002
No	678	1		1		1		1	
Yes	886	1.24 (1.06-1.47)		0.79 (0.65-0.95)		1.19 (1.00-1.40)		0.73 (0.60-0.90)	
Adjuvant radiotherapy			0.008		0.478		0.254		0.667
No	1462	1		1		1		1	
Yes	102	1.46 (1.10-1.93)		1.11 (0.83-1.49)		1.20 (0.88-1.64)		0.93 (0.67-1.29)	
HER2			0.561		0.105		0.898		0.118
Negative (-)	1015	1		1		1		1	
Positive (+)	549	0.95 (0.81-1.12)		1.19 (0.96-1.46)		0.99 (0.83-1.18)		1.19 (0.96-1.48)	

[1]HRs and 95%CI with *P* value based on Cox proportional hazard regression model; [2]Multivariable HRs and 95%CI with *P* value based on Cox proportional hazard regression model. Number of observations in the original data set = 1562. Number of observations used = 1313, owing to the missing value occurring in family history of cancer (249). DFS: Disease free survival; OS: Overall survival.

Table 3 Adjusted[1] hazards ratios for the association between human epidermal growth receptor 2 status and gastric cancer stratified by age, gender, tumor location and TNM stage

Characteristic	DFS		OS	
	HR (95%CI)[1]	$P_{interaction}$ value	HR (95%CI)[1]	$P_{interaction}$ value
Age		< 0.001		0.003
< 60 yr	1.00 (0.69-1.44)		1.09 (0.74-1.60)	
⩾ 60 yr	1.32 (1.02-1.71)		1.28 (0.98-1.68)	
Gender		0.116		0.070
Female	1.10 (0.70-1.73)		1.18 (0.95-1.47)	
Male	1.21 (0.96-1.53)		1.20 (0.88-1.64)	
Tumor location		0.268		0.303
Non-cardia	1.22 (0.91-1.63)		1.20 (0.88-1.64)	
Cardia	1.18 (0.87-1.58)		1.21 (0.88-1.65)	
TNM stage		0.037		0.041
Ⅰ	1.86 (0.98-3.50)		2.03 (0.97-4.23)	
Ⅱ	1.55 (0.94-2.55)		1.75 (1.04-2.94)	
Ⅲ	1.06 (0.83-1.36)		1.03 (0.79-1.33)	

[1]Adjusted HRs and 95%CIs are calculated using models stratified by age, gender, location and TNM stage, with additional adjustment for ethnic, history of family cancer, tumor embolus, neural invasion, adjuvant chemotherapy and adjuvant radiotherapy. Number of observations in the original data set = 1562. Number of observations used = 1313, owing to the missing value occurring in family history of cancer (249). DFS: Disease free survival; OS: Overall survival; TNM: Tumor-node-metastasis.

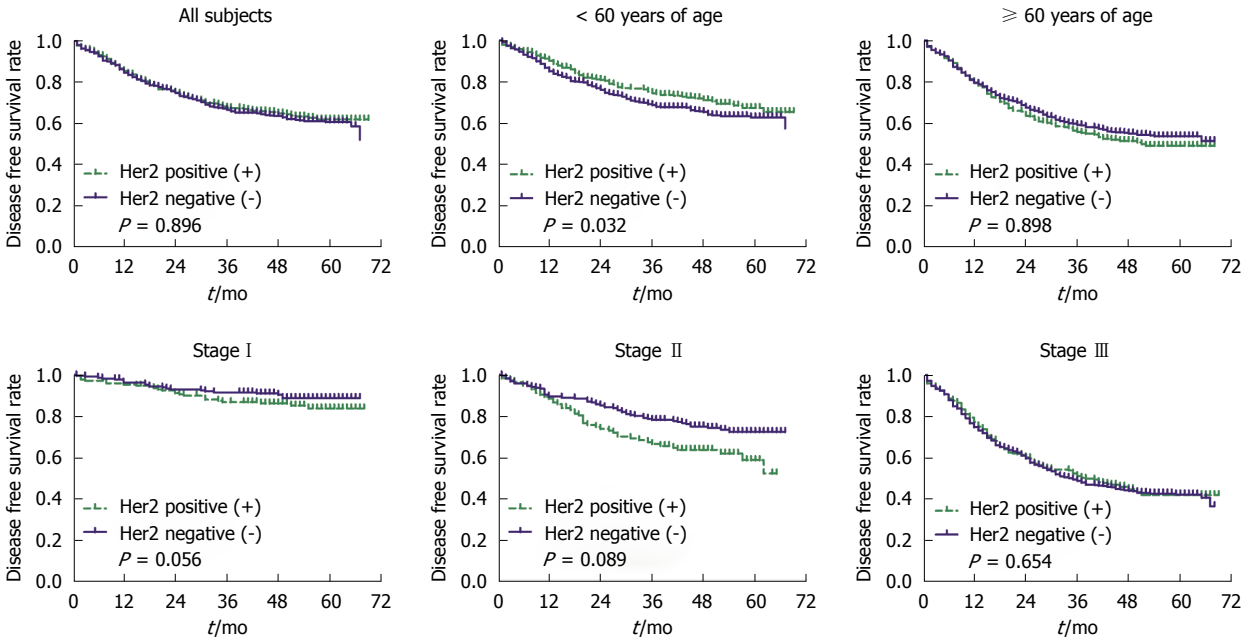

Figure 1 Kaplan-Meier survival curves for disease free survival rate stratified by categorical age (< 60 year *vs* ⩾ 60 year) and pTNM stage without adjustment, respectively. The green dash with plus indicates the positive status of HER2, and the blue solid with plus indicates the negative status of HER2. HER2: Human epidermal growth receptor 2.

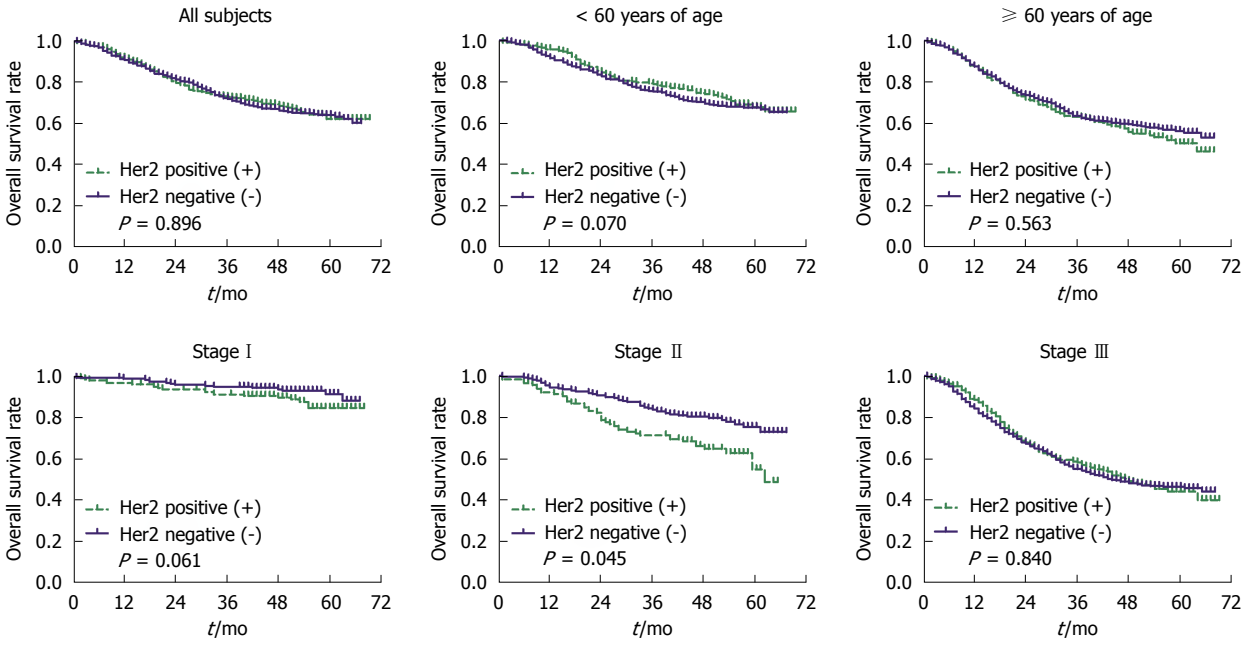

Figure 2 Kaplan-Meier survival curves for overall survival rate stratified by categorical age (< 60 year *vs* ⩾ 60 year) and pTNM stage without adjustment, respectively. The green dash with plus indicates the positive status of HER2, and the blue solid with plus indicates the negative status of HER2. HER2: Human epidermal growth receptor 2.

age (HR = 1.42, 95%CI: 1.18-1.71, $P < 0.001$), pTNM stage (Ⅱ *vs* Ⅰ: HR = 2.61, 95%CI: 1.76-3.87, $P < 0.001$; Ⅲ *vs* Ⅰ: HR = 6.50, 95%CI: 4.57-9.23, $P < 0.001$), neural invasion (HR = 1.51, 95%CI: 1.21-1.89, $P < 0.001$), and adjuvant chemotherapy (HR = 0.79, 95%CI: 0.65-0.95, $P = 0.015$) statistically significantly affected DFS in both univariate and multivariate analyses, that is to say, these factors were independent prognostic factors for DFS among GC patients. Furthermore, categorical age (HR = 1.39, 95%CI: 1.15-1.70, $P < 0.001$), pTNM stage (Ⅱ *vs* Ⅰ: HR = 3.33, 95%CI: 2.14-5.18, $P < 0.001$; Ⅲ *vs* Ⅰ: HR = 8.70, 95%CI: 5.83-12.98, $P < 0.001$), neural invasion (HR = 1.68, 95%CI: 1.33-2.12, $P < 0.001$), and adjuvant chemotherapy (HR = 0.73, 95%CI: 0.60-0.90, $P = 0.002$) were also independent

prognostic factors for OS (Table 2).

DISCUSSION

GC is the fourth most frequently diagnosed cancer and the second leading cause of cancer-related mortality worldwide[19]. However, China accounts for approximately 45% of world's GC cases with an estimated 420489 new cases and 330010 deaths in 2012, and GC remains the second most common GC in both urban and rural areas in China[20]. To date, treatment outcomes for GC remain poor, particularly in patients with inoperable or metastatic disease[21]. Therefore, there is a clear need for effective therapeutic regimes for GC patients. HER2 cancer biomarker testing in GC has been a highly controversial subject, with huge clinical advances taking place in this field while major biomarker mythology discrepancies have persisted[8,22]. HER2 positive GC patients tended to have a more aggressive disease[12,23,24].

Previous studies reported that the prevalence of HER2 amplification is rare and highly heterogeneous in GC with an estimated rate ranging from 4% to 53% owing to the different techniques, methodologies, and scoring systems applied in the studies[4,25-27]. To avoid potential pitfalls in our results, we applied the scoring system approved by the Food and Drug Administration for breast cancer[18] and found that the positive rate of HER2 was approximately 35.08% (95%CI: 32.72-37.45%), which is consistent to the previously reported rates. Although recent studies have been conducted to validate the HER2 scoring system for GC in both IHC analysis and fluorescence in situ hybridization (FISH) analysis, the concordance between FISH and IHC ranged from 88.5% to 93.5%[2,28-30]. In addition, previous reports have demonstrated that a higher rate of HER2 expression is more frequently in intestinal-type than in diffuse-type according to Lauren's classification[8,31] and more common in gastroesophageal junction cancer than in GC[32], but this is not the case in our study.

The role of HER2 as a prognostic factor in GC has been controversial and some initial studies failed to find an association with prognosis[15,26], as in our results. In the post-hoc subgroup analysis of ToGA (Trastuzumab for GC) trial, HER2 positive patients from the Asian population did not reveal a statistically significant improvement of DFS or OS[8]. However, several studies have reported a direct correlation between HER2 expression or HER2 amplification and poorer survival, especially in patients with surgical treatment, gastroesophageal junction cancer and intestinal-type cancer[22,23,33-35]. Even in the Chinese population, Liang *et al*[36] and Zhang *et al*[37] have demonstrated a strong association between HER2 expression and unfavorable survival. In our study, we retrospectively analyzed 1562 patients with GC, all of whom were treated by R0 resection and 35.60% of whom had cardia GC, and found that positive status of HER2 did not affect DFS or OS among the Chinese population. Furthermore, we also conducted subgroup analyses stratified by categorical age, gender, tumor location and pTNM stage. Marginally statistically significant associations were observed between HER2 overexpression and GC in younger patients ($P_{DFS\ Interaction} < 0.001$, $P_{OS\ Interaction} = 0.003$, respectively) and patients with early pTNM stage ($P_{DFS\ Interaction} = 0.037$, $P_{OS\ Interaction} = 0.041$, respectively). Owing to the post-hoc defined group the difference was found by exploratory but not by preplanned analysis, and unrestricted subgroup analysis generates multiple comparisons that dramatically increase the likelihood of detecting a probability value of nominal statistical significance ($P < 0.05$) by chance alone[38]. Therefore, further study should be conducted to confirm these associations in the future.

On the other hand, several limitations should be notable. First and foremost, status of HER2 was tested using IHC analysis only, which may be prone to false negative results especially for IHC 2+ cases, and then lead to bias eventually. Second, sampling errors in specimens of biopsy size may be caused by heterogeneous overexpression of HER2 in GC, which should also be taken into consideration. Third, Lauren classification was also reported to be an important pathological feature of GC patients combined with HER2 status, which indicated a better prognostic factor, while we failed to test the Lauren classification. Finally, 886 (56.72%) and 102 (6.53%) patients received subsequent adjuvant chemotherapy and radiotherapy, respectively, which may lead to a protective effect for patients with GC, despite additional adjustments in this study (Table 2).

In summary, our study provides evidence that HER2 expression was correlated with clinicopathological features of patients with GC undergoing R0 resection, but not associated with unfavorable DFS and OS among the Chinese population.

COMMENTS

Background

Recently, several studies have evaluated the poor prognosis of gastric cancer (GC) with human epidermal growth receptor 2 (HER2) overexpression, whereas the clinical significance of such association remains controversial, especially in the Chinese population. Therefore, we conducted this retrospective study to examine whether the positive status of HER2 can be regarded as an effective prognostic factor for patients with GC undergoing R0 resection among the Chinese population.

Research frontiers

Over the last few years, HER2 has been the most frequently studied molecular biological prognostic factor in various tumors. In GC, the ToGA trial was the first study showing the clinical benefit of trastuzumab in combination with chemotherapy for HER2 positive advanced GC patients. However, a post-hoc subgroup analysis failed to show survival benefits of trastuzumab therapy in Asian populations. Besides, several studies have evaluated the poor prognosis of GC with HER2 overexpression recently, but the clinical significance of such association remains controversial.

Innovations and breakthroughs

The authors conducted this retrospective study to examine whether the positive status of HER2 can be regarded as an effective prognostic factor for patients with GC undergoing R0 resection among the Chinese population, and found the positive status of HER2 based on IHC staining was not related to the survival in patients with GC among the Chinese population.

Applications

In this study, we failed to show survival benefits of trastuzumab therapy in Chinese populations. Therefore, it should be more prudent to use trastuzumab in combination with chemotherapy for HER2 positive GC patients undergoing R0 resection.

Peer-review

The manuscript is very interesting and well written. Considering that the results were retrospectively analyzed, the authors should use the score matched analysis in HER2 positive patients to better explore the prognostic significance of HER2 status in the Chinese population.

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ORIGINAL ARTICLE

Clinical Trials Study

Intestinal-borne dermatoses significantly improved by oral application of *Escherichia coli* Nissle 1917

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Author contributions: Manzhalii E is the responsible author, designed the study and performed the experiments; Hornuss D contributed by his expertise in colonic microbiota and Stremmel W wrote the manuscript.

Institutional review board statement: The study was reviewed and approved by the National Review Board of the Bogomolets National Medical University of Kiev, following the rules of the Helsinki Declaration.

Clinical trial registration statement: This registration policy applies to prospective, randomized, controlled trials only.

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

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Data sharing statement: No additional data are included.

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Abstract

AIM: To evaluate the effect of oral *Escherichia coli* (*E. coli*) Nissle application on the outcome of intestinal-borne dermatoses.

METHODS: In a randomized, controlled, non-blinded prospective clinical trial 82 patients with intestinal-borne facial dermatoses characterized by an erythematous papular-pustular rash were screened. At the initiation visit 37 patients entered the experimental arm and 20 patients constituted the control arm. All 57 patients were treated with a vegetarian diet and conventional topical therapy of the dermatoses with ointments containing tetracycline, steroids and retinoids. In the experimental arm patients received a one month therapy with oral *E. coli* Nissle at a maintenance dose of 2 capsules daily. The experimental group was compared to a non-treatment group only receiving the diet and topical therapy. The primary outcome parameter was improvement of the dermatoses, secondary parameters included life quality and adverse events. In addition the immunological reaction profile (IgA, interleucin-8 and interferon-α) was determined. Furthermore the changes of stool consistency and the microbiota composition over the time of intervention were recorded.

RESULTS: Eighty-nine percent of the patients with acne, papular-pustular rosacea and seborrhoic dermatitis responded to *E. coli* Nissle therapy with significant amelioration or complete recovery in contrast

to 56% in the control arm ($P < 0.01$). Accordingly, in the *E. coli* Nissle treated patients life quality improved significantly ($P < 0.01$), and adverse events were not recorded. The clinical improvement was associated with a significant increase of IgA levels to normal values in serum as well as suppression of the proinflammatory cytokine IL-8 ($P < 0.01$ for both parameters). In the *E. coli* Nissle treated group a shift towards a protective microbiota with predominance of bifidobacteria and lactobacteria ($> 10^7$ CFU/g stool) was observed in 79% and 63% of the patients, respectively ($P < 0.01$), compared to no change in the control group without *E. coli* Nissle. Moreover, the detection rate of a pathogenic flora dropped from 73% to 14 % of the patients in the experimental arm ($P < 0.01$) with no significant change in the control arm (accounting 80% before and 70% after the observation period, $P > 0.05$). Accordingly, stool consistency, color and smell normalized in the *E. coli* Nissle treated patients.

CONCLUSION: *E. coli* Nissle protects the mucus barrier by overgrowth of a favorable gut microbiota with less immunoreactive potential which finally leads to clinical improvement of intestinal borne dermatoses.

Key words: Intestinal-borne dermatoses; *Escherichia coli* Nissle 1917; Immunological response; IgA; Interleukin-8; Interferon-α; Gut microbiota

Core tip: The occurrence of facial dermatoses with erythematous papular-pustular exanthemas is often linked to intestinal inflammation. However, the underlying mechanism remains unclear, and innovative treatment options are missing. Here we show that patients with these dermatoses carry a more aggressive microbiota associated with suppressed serum IgA levels, but increase of the proinflammatory cytokines interleukin-8 and interferon-α. Clinical manifestation, microbiota and inflammatory parameters are significantly improved by application of *Escherichia coli* Nissle. It indicates the usefulness of this probiotic therapy in a neglected patient population in desperate need for effective help.

Manzhalii E, Hornuss D, Stremmel W. Intestinal-borne dermatoses significantly improved by oral application of *Escherichia coli* Nissle 1917. *World J Gastroenterol* 2016; 22(23): 5415-5421 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i23/5415.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i23.5415

INTRODUCTION

Gastrointestinal diseases are often associated with facial dermatoses including acne, rosacea or seborrhoic dermatitis which impair the quality of life of these patients[1-3]. Common feature of these manifestations is an erythematous papular-pustular rash. The digestive system reveals in these cases often infections or an altered microbiota[1,4-8]. Some bacterial genera or species, *e.g.*, bacteroides, firmicutes or bifidobacteria, are predominant in comparison to others like *Escherichia coli* (*E. coli*), lactobacilli and enterococci[4,5,9-13]. Staphylococci, proteus and candida belong to the transient microflora[14]. The composition of the intestinal microbiota is mainly determined by dietary patterns[12]. The function of the microbiota has recently been the focus of scientific interest because it is not only responsible for maintaining a physiological immune response, but also for metabolic processes connected with insulin resistance, obesity and manifestation of fatty liver disease[1,2,8,15-17]. It has also been suggested that an intestinal micro-ecologic imbalance may cause dermatoses induced by an overstimulated immune system[17-19]. This could have therapeutic implications by changing the microbiota towards less aggressive bacterial colonization. One example is the oral application of the *E. coli* strain Nissle 1917 (EcN). By means of special adhesive organelles (by the type F-1A, F-1C and curly fimbriae), the strain has an ability to attach to the mucus membrane of the large intestine and to arrange as microcolonies, forming of a biofilm[20]. Due to the presence of flagella, the bacteria are also mobile, which gives them the advantage of colonizing the colon[21,22]. Therefore, these bacteria were shown to strengthen the mucosal barrier also by interacting with immune modulatory and anti-inflammatory mechanisms[23,24]. *E. coli* Nissle inhibits the growth of Gram-negative anaerobic bacteria by its secretion of antimicrobial substances (microcins) and by siderophores which capture iron and, thus, prevent the growth of certain pathological bacterial strain[16,25]. A postulated overstimulation of the immune system in intestinal disease-related dermatoses by a pathologic microbiota could be identified by elevation of cytokines and chemokines in the circulation[18,26]. Central players are interleukin-8 (IL-8) and α-interferon, which attract mononuclear cells to the site of inflammation to destroy pathogens by activation of the immune system[3]. Before a pathological microbiota invades the organism it has to pass the mucosal barrier. There are several lines of defense which have to be broken[3,26]. The mucus is the first hurdle which has to be taken. Within the mucus there is IgA which is known to inactivate invading bacteria. Since it is secreted from systemic sources, patients with IgA deficiency are prone to intestinal-borne infections[27].

Accordingly, in this study we evaluate the role of IL-8, interferon (INF)-α and IgA as players in the pathogenesis of intestinal disease related dermatoses and the effect of oral administration of *E. coli* Nissle in these conditions.

Table 1 Baseline characteristics of the study population

Characteristic	Experimental arm (n = 37)	Control arm (n = 20)
Sex (women)	63%	61%
Sex (male)	37%	39%
Age (yr)	29 ± 3.1	28 ± 2.5
Smoker	34%	36%
Oral contraception (women)	4%	5%

MATERIALS AND METHODS

In the randomized, controlled, non-blinded, prospective clinical trial 82 patients met the criterion of papular-pustular exanthema with facial manifestation. They were instructed to participate in a clinical trial, informed about the nature of the study and randomized by a closed envelope drawing to the experimental (EA) or control (CA) arm population. Between the evaluation and initiation visit (up to 4 wk interval), 4 and 21 of the participants in the EA and CA groups, respectively, were lost for the study population. The high loss of patients in the control arm was due to the information of the patients that they did not participate in active treatment protocol with *E. coli* Nissle. Thus, finally 37 patients entered the EA and 20 patients constituted the CA group. All included patients underwent physical examinations including the consultation of a dermatologist to verify the diagnosis of the skin dermatoses.

For basic treatment of chronic dermatoses, a diet with predominance of vegetable products was prescribed for all patients. The patients of the control arm (CA) only received standard topical therapy prescribed by a dermatologist, consisting of ointments containing tetracycline, steroids and retinoids (Kremgen® and Lokoid®). The patients of the experimental arm (EA) received a combination treatment which included the standard topical therapy of the dermatoses in combination with oral administration of *E. coli* Nissle 1917 (Mutaflor®): 1 capsule daily for 4 d, then 2 capsules daily for the following month. One capsule of the *E. coli* Nissle 1917 contained 2.5 - 25 × 10^9 live bacteria (CFU). The capsules are resistant to gastric juice and do not disintegrate before they reach the terminal small intestine. The patients were informed about the need to store the medication at a cool place. Follow-up examinations of the dermatoses were performed after a month. The therapeutic effect was estimated according to the dynamics of improvement of the dermatological manifestations. Another criterion was the subjective evaluation of the patients in regard to tolerability and adverse events. Life quality was measured by a scale of 4 index points including: good, acceptable, impaired and not acceptable. For testing of the immunological response in blood, a white blood cell (WBC) differentiation was performed, and the concentration of IL-8 and INF-α was determined by an immunoassay. IgA was quantified by an immunoassay method. The stool of the patients was evaluated in regard to its consistency, color, smell, mucus content and WBC.

Furthermore the stool neutral fat, fatty acid, starch content and presence of muscle fibers were determined[14]. Quantification of bacterial strains was performed by standard techniques[14]. The trial was approved by the local ethical committee.

Ethical permission

All study participants were informed about the study nature and signed a written consent form. The study protocol was approved by the regional committee for research ethics.

Statistical analysis

Statistical analysis performed by using SPSS-20 software. All data in this study were expressed as mean ± SD or percent. The Kolmogorov-Smirnov normality test was used for data distribution analysis. All the values had parametric distribution. Analysis of Variance was applied for multiple comparisons and if the results were significant, a post-hoc Turkey's test was performed. The comparison of the connected values namely the data from the same patient before and after treatment was done using the Student's *t*-test for paired samples. The differences between groups were considered significant at $P < 0.05$.

RESULTS

Out of 123 patients with dermatoses primarily evaluated, 82 patients revealed a papular-pustular exanthema with facial manifestation. Of these, 57 patients agreed to participate in the study and were finally included in the trial with an age range from 18 to 42 years, a disease duration range from 1 to 10 years, and a gender distribution of 35 women and 22 men. The experimental arm (EA) consisted of 37 and the control arm (CA) of 20 patients (Table 1).

All patients revealed the predominant feature of erythema in conjunction with papular-pustular elements. Twenty-two percent out of the patients were diagnosed with acne, 36% with papular-pustular rosacea and 57% with seborrhoic dermatitis. In 10% of the cases the entire facial skin was involved. Concerning the primary end point of the trial, the improvement of the dermatologic features was significantly greater in the EA compared to the CA group ($P < 0.01$). After one month, in the EA group 32% showed recovery and 57% significant amelioration (11% simple amelioration), whereas in the CA group only 17% revealed recovery and 39% significant amelioration (32% simple amelioration and 12% no change) (Figure 1).

The resolution of clinical manifestations of the inflammatory process of the facial skin occurred in

Table 2 Interferon-α, interleukin-8 and IgA in serum before and after treatment

Arms of the patients	INF-α, pg/mL		IL-8, pg/mL		IgA, g/L	
	Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment
Experimental arm (n = 37)	8.07 ± 2.97	3.73 ± 2.88[d, f]	30.8 ± 4.42	17.1 ± 0.65[d, f]	0.45 ± 0.05[b]	0.71±0.22[d, f]
Control arm (n = 20)	6.88 ± 1.77	6.12 ± 1.53	27.0 ± 2.72	25.2 ± 1.23[b]	0.35 ± 0.9	0.54 ±0.05[d]

[b]P < 0.01 and [d]P < 0.001 significant difference before *vs* after treatment; [f]P < 0.01 *vs* control arm after treatment. INF: Interferon; IL: Interleukin.

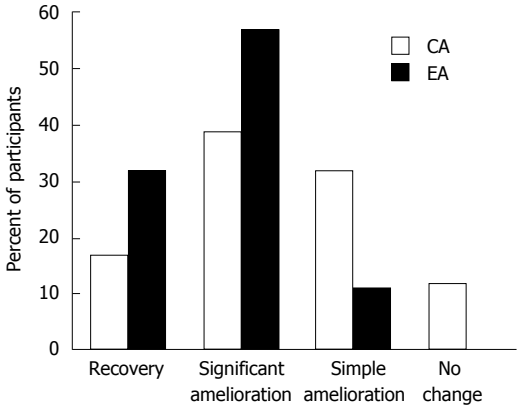

Figure 1 Improvement of dermatological features in both groups after 1 mo. Illustrated are the percentages of patients with facial dermatoses in the control arm (CA) and experimental arm (EA) over the time of the trial. They are categorized according to the degree of change in clinical manifestation.

the reversed order of their development. Initially, edema and swelling decreased, later papular rush and erythema faded as well as the formation of new papulae and pustulae discontinued. This was followed by disappearance of crusts in the area of the lesions, and nodular eruptions gradually flattened (Figure 2 as an example).

All patients in both groups tolerated the treatment very well, and adverse events were not recorded. All patients in the EA showed an increase of life quality by 1.7 ± 0.6 index points (< 0.01) revealing an acceptable or good condition in all patients of the EA group in contrast to the CA group with an overall unchanged impaired life quality (P > 0.05). Accordingly, *E. coli* Nissle showed high therapeutic efficacy in addition to good tolerability and absence of serious adverse reactions reported by the patients.

Elevated INF-α values showed a trend towards reduction in the EA patient population after therapy, but did not reach statistical significance. Low serum IgA levels were initially recorded in the EA and CA group. After treatment, the IgA level was normalized only in the EA arm (Table 2 and Figure 3). The same is true for the elevation of IL-8 cytokine levels which were normalized after treatment in the EA patient group. This is probably due to the immunomodulatory properties of *E. coli* Nissle 1917 which decreases the level of newly activated T-lymphocytes migration into the focus of inflammation. Accordingly, also the lymphocytosis disappeared in 78% of the EA group, whereas it improved in the CA group only in 42 % of the patients (Table 2 and Figure 3).

In regard to stool appearance, 82% of the patients initially had loose stools of grey color, sticky consistency with strong smell and mucus in large amounts. After treatment, 71% of participants in the EA had a formed stool of typical color and smell, and only small amounts of mucus.

Before treatment, bacteriological stool culture showed a decrease in the number of bifidobacteria and lactobacteria in both patient groups but an increase in potential pathogenic bacteria, *i.e.,* staphylococci, yeasts, bacteroides, proteus, citrobacter and klebsiellae (Table 3).

After therapy with *E. coli* Nissle (EA), an increase of bifidobacteria and lactobacteria in stool cultures was noted (P < 0.01 for both species). There was a significant decrease recorded in the number of staphylococci, yeasts, bacteroides, proteus, citrobacter, klebsieallae in 59% of the EA-patients as compared to no change in the CA-group (P < 0.01) (Table 3).

DISCUSSION

The mechanism by which intestinal diseases induce related dermatoses is obscure. Here we show that intestinal-borne dermatoses are accompanied by a shift towards a more aggressive intestinal microbiota spectrum. Due to their potential to invade the mucosal barrier, they activate the immune system with elevation of IL-8 and interferon-α. This could be the reason for induction of dermatoses because they attract mononuclear cells to preformed lesions of the skin, leading to inflammation. The dermatoses are significantly improved after oral application of *E. coli* Nissle. Whether the low IgA levels initially recorded in the patient population are the consequence or origin of invasion of pathogenic bacteria remains to be determined. However, the consumption of IgA within the mucus seems more likely because it returns to normal values after the treatment course.

In addition to the observed changes of the microbiota distribution, the biofilm of *E. coli* Nissle per se may also have an impact on stool consistency. It may be due to an effect on motility as well as the

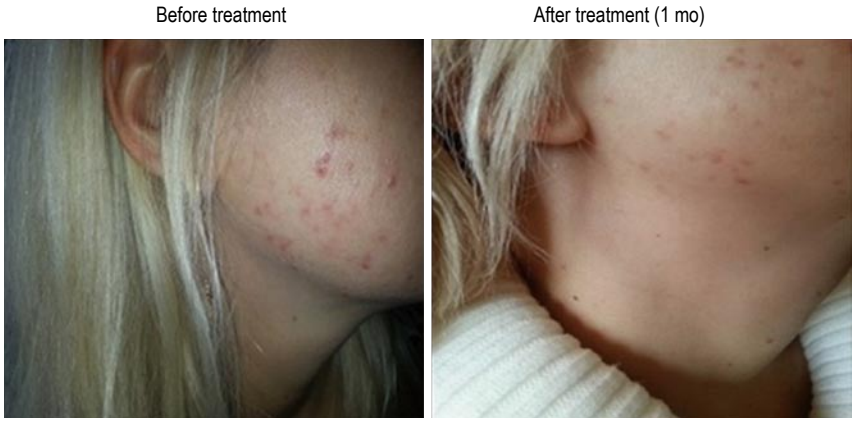

Figure 2 Female patient; EA group before and one month after treatment. Clinical example of a patient treated with *E. coli* Nissle. The facial popular-pustular exanthema was significantly improved over the 1 mo treatment period.

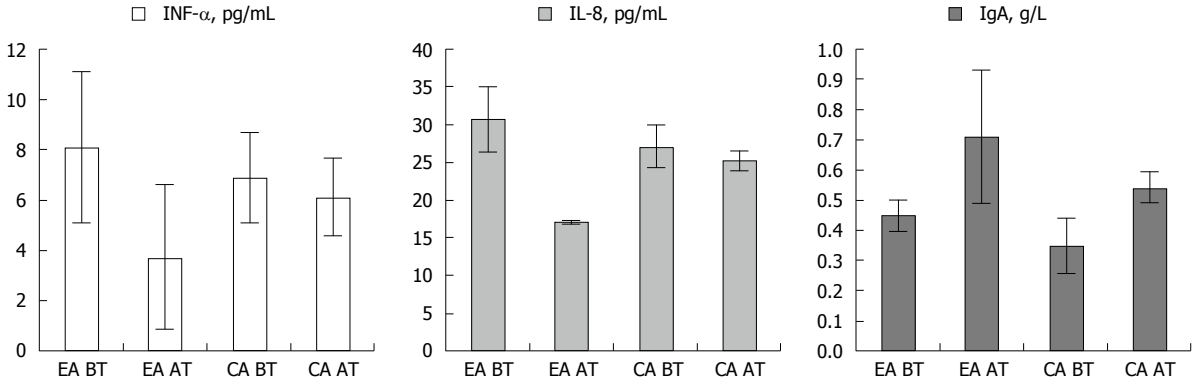

Figure 3 Improvement of serum levels of INF-α, IL-8 and IgA in experimental arm before (EA BT) and after treatment (EA AT), and control arm before (CA BT) and after treatment (CA AT). Serum INF-α, IL-8 and IgA levels over the trial course. Illustrated are the values of INF-α , IL-8 and IgA in the experimental arm before (EA BT) and after treatment (EA AT) and the control arm before (CA BT) and after treatment (CA AT).

Table 3 Changes of the flora after treatment with *E. coli* Nissle *n* (%)

Microflora characteristics	Experimental arm (n = 37)		Control arm (n = 20)	
	Before treatment	After treatment	Before treatment	After treatment
Bifidobacteria > 10^7 CFU/g	5 (14)	29 (79)[b]	3 (15)	3 (15)
Normal < 10^7 CFU/g	26 (70)	7 (19)[b]	15 (75)	14 (70)
Below normal absent	6 (16)	1 (2)[b]	2 (10)	3 (15)
Lactobacteria > 10^7 CFU/g	3 (8)	23 (63)[b]	3 (15)	2 (10)
Normal < 10^7 CFU/g	27 (73)	13 (35)[b]	15 (75)	15 (75)
Below normal absent	7 (19)	1 (2)[b]	2 (10)	3 (15)
Pathogenic microflora	27 (73)	5 (14)[b]	16 (80)	14 (70)

[b]$P < 0.01$ significant difference before *vs* after treatment.

functionality of the mucus barrier. The production of short-chain fatty acids increases the nutritional state of the mucus and thus its capability to absorb water[14,20]. This improves the motility as well as the absorption of water and sodium. All of this helps to form a more consolidated stool. More importantly, because the mucosal barrier is strengthened, pathogens cannot easily penetrate and, thus, the *E. coli* Nissle application prohibits systemic activation of the immune system eventually inducing intestinal-borne dermatoses.

In conclusion, we report that *E. coli* Nissle is very effective to treat intestinal-borne chronic dermatoses. It shows good tolerability and no adverse events. The mode of action relates to change of the intestinal microbiota towards less aggressive bacteria. This in turn ameliorates the immune response characterized by a normalization of IgA and IL-8. Thus, it represents a treatment option for patients with intestinal borne dermatosis.

COMMENTS

Background

Intestinal-borne dermatoses have unknown etiology and there is a medical need for their therapy. The recent observation that the gut microbiota has impact on the immune system guided us to the question whether this induces

dermatoses and whether microbiota modulation may be of therapeutic use.

Research frontiers

The present study focuses on etiology and therapy of intestinal dermatoses as a neglected field in gastroenterology, although a large number of patients suffer from this entity. It covers the areas of microbiota and the associated systemic immune response.

Innovations and breakthroughs

It is an innovative approach to link the gut microbiota to the pathogenesis of intestinal-borne dermatoses. The fact that modulation of the microbiota by application of *Escherichia coli* (*E. coli*) Nissle 1917 improves the dermatoses was unexpected and opens a new avenue of therapy for these patients in need.

Applications

The study provides a rationale for the therapy of intestinal-borne dermatoses. Indeed it is shown that *E. coli* Nissle 1917 improves these dermatoses by suppressing the intestinal microbiota-triggered immune response. It will open avenues of new therapies also with other microbiota-modulating regimens. The study may also stimulate basic research to unravel the interaction of the gut microbiota and the immune system.

Terminology

The paper deals with different intestinal-borne dermatoses, the adaptive immune response mechanism, the composition of the gut microbiota in regard to protective and aggressive bacterial colonization and the biological activity of *E. coli* Nissle 1917.

Peer-review

The manuscript is very interesting for the readers.

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ORIGINAL ARTICLE

Observational Study

Endocan-expressing microvessel density as a prognostic factor for survival in human gastric cancer

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Author contributions: Chang Y designed the research and drafted and revised the paper; Niu W, Lian PL and Wang XQ performed the research; Meng ZX and Liu Y searched the literature and analyzed the data; and Zhao R revised the paper and approved the final version.

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Abstract

AIM: To investigate the expression of endocan in tumour vessels and the relationships between endocan and the expression of vascular endothelial growth factor (VEGF) and prognosis in gastric cancer.

METHODS: This study included 142 patients with confirmed gastric cancer in a single cancer centre between 2008 and 2009. Clinicopathologic features were determined, and an immunohistochemical analysis of endocan-expressing microvessel density (MVD) (endocan-MVD), VEGF and vascular endothelial growth factor receptor 2 (VEGFR2) was performed. Potential relationships between endocan-MVD and clinicopathological variables were assessed using a Student's t-test or an analysis of variance test. Spearman's rank correlation was applied to evaluate the relationship between endocan-MVD and the expression of VEGF/VEGFR2. Long-term survival of these patients was analysed using univariate and multivariate analyses.

RESULTS: Positive staining of endocan was observed in most of the gastric cancer tissues (108/142) and in fewer of the normal gastric tissues. Endocan-MVD was not associated with gender or histological type ($P > 0.05$), while endocan-MVD was associated with tumour size,

Borrmann type, tumour differentiation, tumour invasion, lymph node metastasis and TNM stage ($P < 0.05$). According to the Spearman's rank correlation analysis, endocan-MVD had a positive correlation with VEGF ($r = 0.167$, $P = 0.047$) and VEGFR2 ($r = 0.410$, $P = 0.000$). The univariate analysis with a log-rank test indicated that the patients with a high level of endocan-MVD had a significantly poorer overall survival rate than those with a low level of endocan-MVD (17.9% *vs* 64.0%, $P = 0.000$). The multivariate analysis showed that a high level of endocan-MVD was a valuable prognostic factor.

CONCLUSION: Endocan-MVD significantly correlates with the expression of VEGF and VEGFR2 and is a valuable prognostic factor for survival in human gastric cancer.

Key words: Endocan; Microvessel density; Vascular endothelial growth factor; Gastric cancer; Survival

Core tip: Angiogenesis plays an important role in the progression of gastric cancer. In the present study, we first found that endocan-expressing microvessel density (MVD) (endocan-MVD) had a positive correlation with vascular endothelial growth factor (VEGF) and VEGFR2 in gastric cancer tissues. Patients with a high level of endocan-MVD had a significantly poorer overall survival rate than those with a low level of endocan-MVD. Based on our research, we suggest that endocan-MVD may act as a valuable prognostic factor for survival in patients with gastric cancer.

Chang Y, Niu W, Lian PL, Wang XQ, Meng ZX, Liu Y, Zhao R. Endocan-expressing microvessel density as a prognostic factor for survival in human gastric cancer. *World J Gastroenterol* 2016; 22(23): 5422-5429 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i23/5422.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i23.5422

INTRODUCTION

Gastric cancer, a malignancy that remains the second leading cause of cancer-related deaths worldwide and causes high morbidity and mortality, is one of the most common malignancies in the world[1]. Compared to developed countries, developing countries have higher morbidity and mortality rates. Worldwide, more than 70% of new cases of gastric cancer and related deaths occur in developing countries, such as in Eastern Europe, East Asia and South America[2]. In particular, in China, there are approximately 400000 new cases annually, which account for 42% of the total global cases, and the death toll in China is 300000[3]. Therefore, identifying a new potential therapeutic target can benefit diagnosis and treatment. It is critical to investigate new factors that are closely associated with the initiation and development of gastric cancer.

Endocan, previously called the endothelial cell specific molecule-1, which is a new member of the proteoglycan family, plays an extremely important role in the transformation, survival, proliferation, invasion, angiogenesis and metastasis of tumours[4-7]. Previous *in vivo* and *in vitro* studies have demonstrated that endocan is overexpressed in human tumours, such as in colon cancer, kidney cancer and prostate cancer[8-10]. Consistently, elevated blood levels of endocan have also been observed in patients with lung cancers[11]. In particular, in liver cancer and gastric cancer[12,13], serum levels of endocan are significantly changed after treatment; thus, endocan is a biomarker of tumour progression as well as a validated therapeutic target.

It has been extensively reported that the secretion of endocan by cultured endothelial cells is strongly upregulated in the presence of proangiogenic molecules such as vascular endothelial growth factor (VEGF)[14]. Endocan has been shown to be activated by VEGF through the PKC and PI3K signalling pathways in human umbilical vein endothelial cells, and this process can be inhibited by PI3K pathway blockers[15]. Furthermore, the blockade of VEGFR2 by specific antibodies completely prevented VEGFC-mediated induction of endocan expression in endothelial cells[16]. Data obtained from cancer tissue samples have shown that endocan can be visualized in newly growing tumour vessels of endothelial cells. Thus, endocan-expressing microvessel density (MVD) is likely to be valuable for assessing the prognosis of malignancy. Vascular endocan, as a biomarker of neoangiogenesis, is found inside the vessels in different types of tumours, such as colon, kidney, prostate and brain cancers, but not in gastric cancer[8-10,17]. Thus, endocan immunolabelling was explored in this study to understand the progression of gastric cancer.

The aim of this study was to examine the expression of endocan in gastric cancer vessels, to investigate the relationship between endocan-MVD and the expression of VEGF and VEGFR2, and to determine whether endocan-MVD could be used to predict the outcomes of patients with gastric cancer.

MATERIALS AND METHODS

Patients

A total of 142 patients with gastric cancer who had undergone gastrectomy were enrolled in this study between January 2008 and April 2009 at the Qilu Hospital of Shandong University. All patients had undergone primary curative gastrectomy, and none of them had received chemotherapy or radiotherapy before surgery. Meanwhile, 142 specimens of adjacent tissues were obtained from these patients to serve as the paired controls. The tissues were embedded in paraffin after 16 h of formalin fixation. Of the patients

Table 1 Correlation between endocan-microvessel density and clinicopathological features and vascular endothelial growth factor/vascular endothelial growth factor receptor 2 expression in gastric cancer

Clinicopathologic feature	*n*	Endocan-MVD	*P* value[1]
Gender			0.27
Male	93	18.19 ± 8.240	
Female	49	19.79 ± 8.203	
Age (yr)			0.32
< 60	78	19.36 ± 8.436	
⩾ 60	64	18.00 ± 7.982	
Tumour size (cm)			0.000[a]
< 5	79	16.48 ± 9.170	
⩾ 5	63	21.58 ± 5.799	
Borrmann type			0.000[a]
Ⅰ	18	11.33 ± 3.970	
Ⅱ	15	11.93 ± 4.651	
Ⅲ	81	21.17 ± 8.122	
Ⅳ	28	20.14 ± 7.437	
Histology			0.25
Adenocarcinoma	118	19.11 ± 8.183	
Signet ring cell carcinoma	8	16.55 ± 2.554	
Mixed carcinoma	15	17.13 ± 10.602	
Differentiation			0.000[a]
High/moderate	36	11.66 ± 4.623	
Low/undifferentiated	106	21.15 ± 7.803	
Tumour invasion			0.000[a]
T1	2	6.33 ± 0.577	
T2	30	10.30 ± 4.822	
T3	4	16.33 ± 0.577	
T4	106	21.56 ± 7.168	
Lymph node metastasis			0.000[a]
N0	33	12.60 ± 7.314	
N1	21	17.521 ± 5.662	
N2	30	16.96 ± 7.420	
N3	58	23.62 ± 7.053	
TNM stage			0.000[a]
Ⅰ	15	8.80 ± 3.764	
Ⅱ	18	11.38 ± 5.679	
Ⅲ	99	20.85 ± 7.331	
Ⅳ	10	26.10 ± 2.960	
VEGF expression			0.001[a]
Negative	58	16.32 ± 6.227	
Positive	84	20.43 ± 9.015	
VEGFR2 expression			0.000[a]
Negative	66	12.48 ± 5.239	
Positive	76	24.19 ± 6.222	

[1]Student *t*-test; [a]$P < 0.05$, statistically significant. VEGF: Vascular endothelial growth factor; VEGFR2: VEGF receptor 2; MVD: Microvessel density.

enrolled in this study, 93 (65.4%) were male and 49 (34.5%) were female, with a median age of 56.8 years (ranging from 24 to 79 years). The cut-off date for follow-up was April 2015, and the median follow-up duration was 39 mo (ranging from 6 to 72 mo). The clinicopathological features of the patients examined included gender, age, Borrmann type, tumour size, tumour histological morphology, tumour differentiation (according to the WHO classification for gastric cancer in 2000), tumour invasion, lymph node metastasis, TNM stage (TNM 7th edition by the American Joint Committee on Cancer) and vascular invasion. This study was approved by the Ethics Committee of Qilu Hospital, Shandong University, and all study participants signed an informed consent form. The clinicopathological characteristics of the patients are summarized in Table 1.

Immunohistochemistry

Paraffin-embedded tissues containing primary tumours and paired controls were obtained from the archives of the Department of Pathology of our hospital. Successive sections at 1 mm intervals from each paraffin block were used for evaluation. Anti-endocan mouse monoclonal antibody (ab56914, Abcam, Cambridge, MA, United States), anti-VEGF mouse monoclonal antibody (sc-7269, Santa Cruz Biotechnology Inc., Santa Cruz, CA, United States) and anti-VEGFR2/Flk-1 mouse monoclonal antibody (sc-6251, Santa Cruz Biotechnology Inc., Santa Cruz, CA, United States) were used for the immunohistochemical staining of endocan, VEGF and VEGFR2, respectively.

The immunohistochemical staining was performed using the streptavidin peroxidase complex method. The slides were deparaffinized with xylene and washed with PBS. After antigen retrieval, the slides were incubated with 0.3% blocking serum for 30 min at 37 ℃ to reduce nonspecific binding. The sections were incubated overnight at 4 ℃ with primary antibody against endocan (dilution: 1:100), VEGF (dilution: 1:150) or VEGFR2 (dilution: 1:150). The tissue sections were rinsed in TBS and then detected using biotinylated goat anti-mouse immunoglobulin as secondary antibody. After rinsing with TBS, the sections were incubated with 3,3'-diaminobenzidine solution until the desired staining was achieved. The slides were counterstained with haematoxylin, dehydrated, cleared and mounted. Negative controls were created by omitting the primary antibodies.

The immunohistochemical staining was evaluated independently by two pathologists who were blinded to the clinical information and the nature of the specimens. Quantitative analysis of MVD was performed in the sections that were stained for endocan. The most vascularized areas within the tumours ("hot spots") were chosen at low magnification (× 40), and the vessels were counted at representative high magnification (× 400). All brown-stained endothelial cells that were clearly separated from connective tissue elements were considered microvessels. MVD was counted in three fields and was recorded as a total number per unit area. Endocan-MVD was divided into four groups (0, 1, 2 and 3) based on quartiles of endocan-MVD numbers, which were 12.51, 18.86 and 25.88, respectively. For all cases, the scores (0) and (1) were defined as a low level of endocan-MVD, and (2) and (3) as a high level of endocan-MVD.

The immunohistochemical results of VEGF and VEGFR2 were classified according to the number of positive cells as follows: (-), no cell was stained; (+), < 25% of cells were stained; (++), 25%-50% of cells were stained; and (+++), > 50% of cells

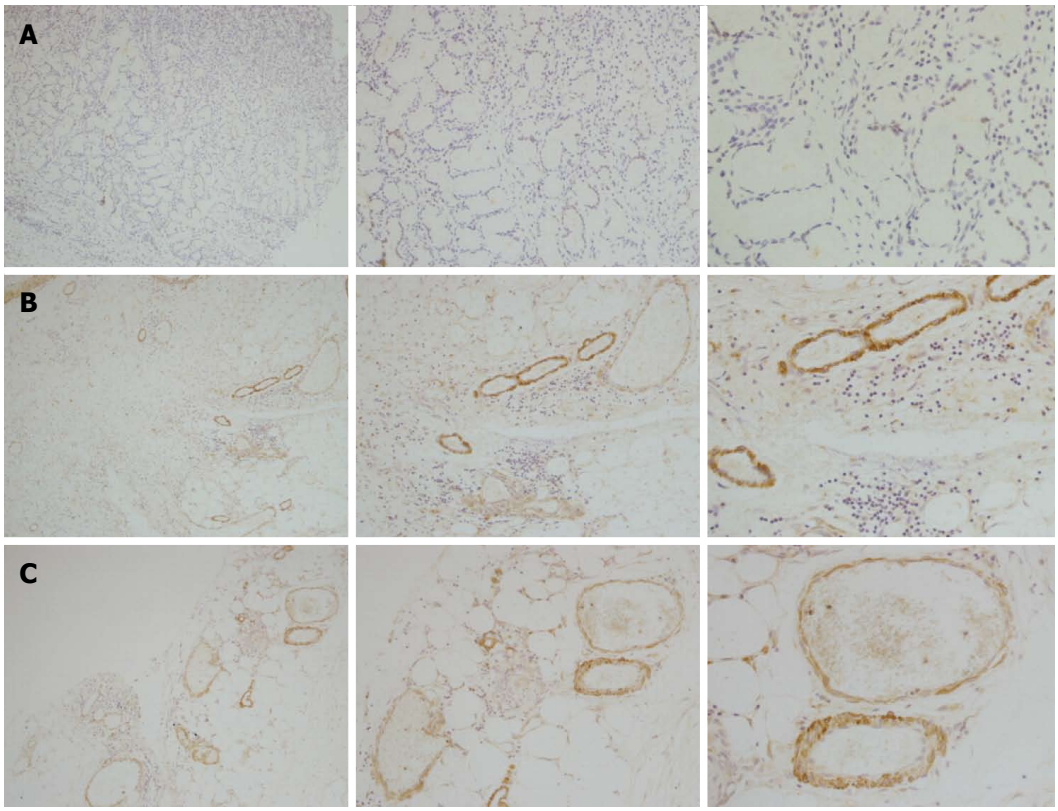

Figure 1 Immunohistochemical staining of endocan in gastric cancer. Positive staining of endocan was observed in most of the endothelial cells of the tumour vessels, but less staining was observed in normal gastric tissues (A); In the peritumour vascular endothelium (B); positive endocan was more compact than that in the tumour centres (C).

were stained. For all cases, the scores (-) and (+) were defined as the negative expression of VEGF and VEGFR2, and (++) and (+++) as positive expression.

Statistical analysis

The SPSS program (version 16, SPSS Inc., Chicago, IL, United States) was used for the statistical analyses. Continuous quantitative data with a normal distribution are expressed as the mean ± SD. Potential relationships between endocan-MVD and clinicopathological variables were assessed using a Student's *t*-test or an analysis of variance test, as appropriate. Spearman's rank correlation was applied to evaluate the relationship between endocan-MVD and the expression of VEGF/VEGFR2. A Kaplan-Meier analysis was used to assess patient survival. We conducted a univariate analysis of the prognostic factors with a log-rank test and a multivariate analysis with a Cox's regression model. $P < 0.05$ was considered statistically significant.

RESULTS

Expression of endocan in gastric cancer tissues

Positive staining of endocan was observed in most of the gastric cancer tissues (108/142) and in fewer of the normal gastric tissues (Figure 1A and B). The expression of endocan was also found in the endothelial cells of tumour vessels. In tumour centres, endocan-expressing endothelial cells of tumour vessels were observed in 78% (85/108) of the gastric cancer tissues stained with the endocan antibody (Figure 1C). In the peritumour vascular endothelium, positive endocan staining was more compact and was observed in most cases of gastric cancer tissues stained with the endocan antibody (100/108; Figure 1B). The endocan-MVD in gastric cancer tissues was 18.8 ± 8.1 and ranged from 4 to 44.

Relationship between endocan-MVD and clinicopathological features

Endocan-MVD was significantly correlated with tumour size, Borrmann type, tumour differentiation, tumour invasion, lymph node metastasis, TNM stage and the expression of VEGF and VEGFR2, whereas it was not correlated with gender, age or histology. These data are summarized in Table 1.

Relationship between endocan-MVD and the expression of VEGF and VEGFR2

VEGF and VEGFR2 were dispersed granularly within the cytoplasm of the tumour cells (Figure 2). Among the total 142 gastric cancer specimens, VEGF overexpression was detected in 84 (59.1%), and VEGFR2 was overexpressed in 76 (53.5%). A

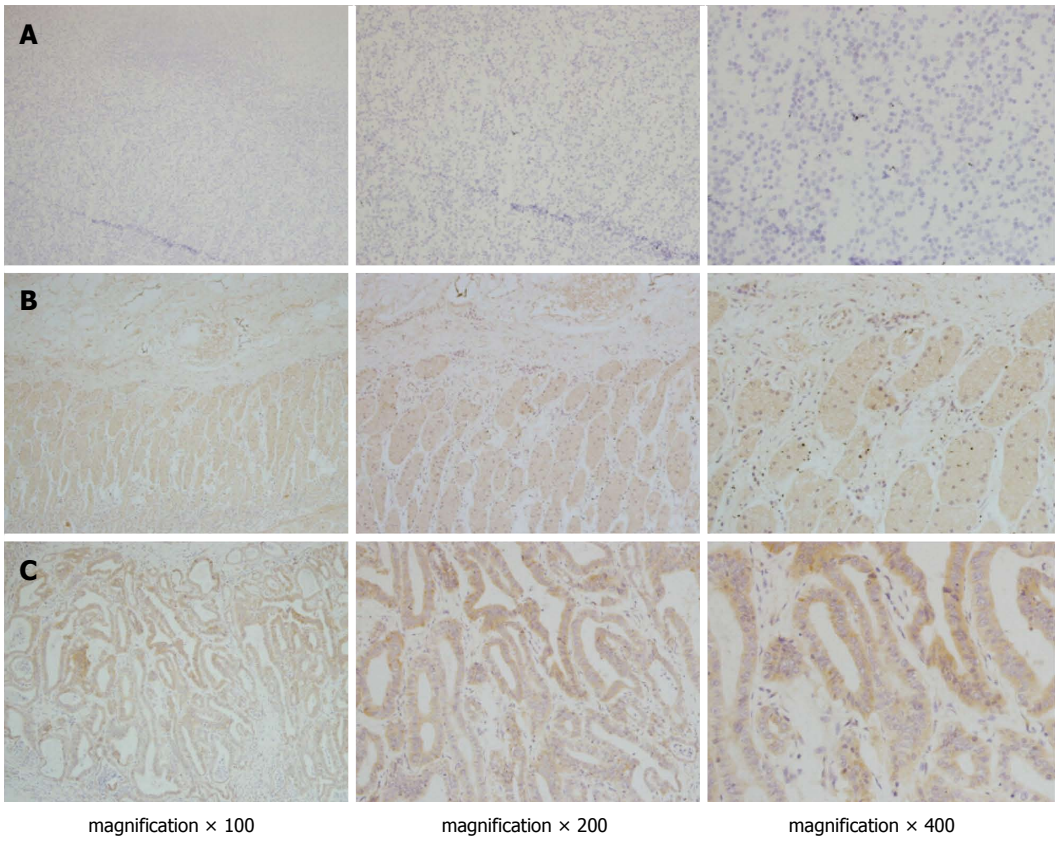

Figure 2 Immunohistochemical staining of vascular endothelial growth factor and vascular endothelial growth factor receptor 2 in gastric cancer. Positive staining of VEGF (B) and VEGFR2 (C) was observed in most gastric cancer tissues, but less staining was observed in normal gastric tissues (A). VEGF: Vascular endothelial growth factor; VEGFR2: VEGF receptor 2.

Table 2 Correlation between endocan-microvessel density and vascular endothelial growth factor expression in gastric cancer (r = 0.167, P = 0.047), and between endocan-microvessel density and vascular endothelial growth factor receptor 2 expression in gastric cancer (r = 0.410, P = 0.000)

Endocan-MVD[1]	Total	VEGF[2]				VEGFR2[2]			
		-	+	++	+++	-	+	++	+++
0	34	14	2	15	3	20	12	2	0
1	41	15	8	12	6	12	14	10	5
2	40	5	8	14	13	2	5	12	21
3	27	2	4	8	13	0	1	4	22
Total	142	36	22	49	35	34	32	28	48

[1]Endocan-MVD was divided into four groups (0, 1, 2, and 3) based on quartiles of Endocan-MVD number, which were 12.51, 18.86 and 25.88, respectively; [2]VEGF and VEGFR2 were divided as follows: (-), no cell stained; (+), < 25% of cells stained; (++), 25%-50% of cells stained; (+++), > 50% of cells stained. VEGF: Vascular endothelial growth factor; VEGFR2: VEGF receptor 2; MVD: Microvessel density.

significant association was found between endocan-MVD and the expression of VEGF or VEGFR2 (Spearman's rank correlation analysis, r = 0.167, P = 0.047 and r = 0.410, P = 0.000, respectively; Table 2).

Univariate and multivariate analyses of prognosis variables

The 142 patients were divided into a low endocan-MVD group (75/142) and a high endocan-MVD group (67/142) using the median value of endocan-MVD (*i.e.*, 18.8). The univariate analysis with a log-rank test indicated that the patients with a high endocan-MVD had a significantly poorer overall survival rate than those with a low endocan-MVD (17.9% *vs* 64.0%, P = 0.000; Figure 3). Tumour size, Borrmann type, differentiation, tumour invasion, lymph node metastasis, TNM stage and the expression of VEGF and VEGFR2 were significantly associated with the overall survival rate, whereas the other clinicopathologic features were not (Table 3).

Parameters with P values < 0.05 in the univariate analysis were included in the multivariate analysis using a Cox proportional hazards model. It was revealed that differentiation, TNM stage, endocan-MVD and the expression of VEGF and VEGFR2 were significant prognostic factors in these patients (Table 4).

DISCUSSION

Angiogenesis plays an important role in the invasion, growth and metastasis of most tumours in which a number of cytokines are now known to be involved[18]. Among these cytokines is an essential factor for angiogenesis, which is known as VEGF. VEGF not only stimulates the division and migration of vascular

Table 3 Univariate analysis of the correlation between clinicopathologic features and overall survival of 142 patients with gastric cancer

Clinicopathologic feature	*n*	5-yr survival rate (%)	*P* value[1]
Gender			0.280
Male	93	44.08	
Female	49	38.77	
Age (yr)			0.580
< 60	78	41.02	
⩾ 60	64	43.75	
Tumour size (cm)			0.000[a]
< 5	79	62.02	
⩾ 5	63	17.46	
Borrmann type			0.000[a]
Ⅰ + Ⅱ	33	78.78	
Ⅲ + Ⅳ	109	31.19	
Histology			0.260
Adenocarcinoma	118	40.67	
Others	23	52.17	
Differentiation			0.000[a]
High/moderate	36	69.44	
Low/undifferentiated	106	33.01	
Tumour invasion			0.000[a]
T1-2	32	84.37	
T3-4	110	30.00	
Lymph node metastasis			0.003[a]
N0-1	54	57.40	
N2-3	88	32.95	
TNM stage			0.000[a]
Ⅰ-Ⅱ	33	84.84	
Ⅲ-Ⅳ	109	29.35	
VEGF expression			0.003[a]
Negative	58	56.89	
Positive	84	32.14	
VEGFR2 expression			0.000[a]
Negative	66	68.18	
Positive	76	19.73	
Endocan-MVD			0.000[a]
Low	75	64.00	
High	67	17.91	

[1]Log-rank test; [a]$P < 0.05$, statistically significant. VEGF: Vascular endothelial growth factor; VEGFR2: VEGF receptor 2; MVD: Microvessel density.

Table 4 Multivariate analysis of the correlation between clinicopathologic features and overall survival of 142 patients with gastric cancer

Factor	*P* value	HR	95%CI
Tumour size (cm)	0.247	1.407	0.789-2.508
Borrmann type	0.388	1.345	0.686-2.636
Differentiation	0.036[a]	3.52	1.087-11.394
Tumour invasion	0.681	1.465	0.237-9.066
Lymph node metastasis	0.75	1.119	0.560-2.235
TNM stage	0.000[a]	4.189	1.450-12.102
VEGF expression	0.001[a]	2.827	1.508-5.302
VEGFR2 expression	0.020[a]	3.572	1.218-10.474
High endocan-MVD	0.047[a]	2.111	1.008-4.419

[a]$P < 0.05$, statistically significant. VEGF: Vascular endothelial growth factor; VEGFR2: VEGF receptor 2; MVD: Microvessel density.

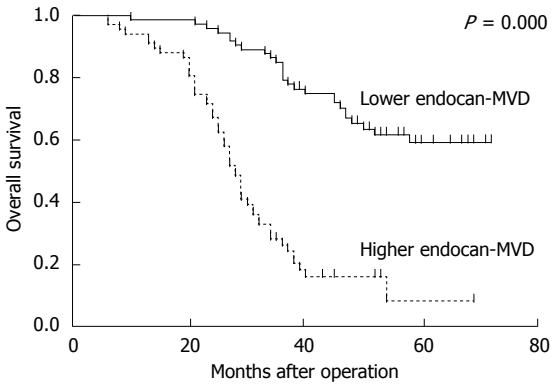

Figure 3 Overall survival curves of the patients in the high and low endocan-microvessel density groups. Patients with high endocan-MVD had a significantly poorer overall survival rate than those with low endocan-MVD (17.9% *vs* 64.0%, *P* = 0.000). MVD: Microvessel density.

endothelial cells but can also induce new vessels, which are thin and susceptible to the invasion of cancer, leading to distant metastases[19]. Microvascular density is used as an objective evaluation index for tumour angiogenesis. Previous studies have shown that endocan is expressed in endothelial cells in colon cancer and kidney cancer, and endocan-MVD is associated with the prognosis of tumours[8-10]. Therefore, in this paper, we performed further research on the relationship between endocan-MVD and VEGF/VEGFR2 and the prognosis of patients with gastric cancer to provide a new therapy for patients with gastric cancer.

Endocan is the product of a gene that is located in the proximal region of the long arm of chromosome 5 (5q11.2)[4]. Recent evidence has implied that endocan plays important roles in several pathophysiological processes, including inflammatory disorders and tumour progression, and in the regulation of major cellular processes, such as adhesion, migration and angiogenesis[4-7]. Numerous studies have focused on endocan expression in tumour tissues, and it is deemed as a new tumour prognostic marker because of its expression in serum[20,21]. However, our studies paid more attention to the expression of endocan in tumour vessels, as well as the relationship between the expression of endocan and tumour clinical features. We have confirmed the expression of endocan in the endothelial cells of tumour centre vessels, and positive endocan showed denser expression in the peritumour vascular endothelium. This showed that endocan is crucial in gastric cancer angiogenesis.

To test whether endocan-MVD can predict the prognosis of gastric cancer after resection, we used immunohistochemical staining to determine endocan expression in tumour specimens and correlated our findings with available follow-up information. We found that positive staining of endocan was preferentially detected in gastric cancer vessels but not in normal gastric tissues. Endocan-MVD in the peritumour vascular endothelium was higher than that in the tumour centre. The data from the current study

indicated that endocan-MVD in gastric cancer was significantly associated with tumour size, Borrmann type, differentiation, tumour invasion, lymph node metastasis, TNM stage and the expression of VEGF and VEGFR2. By analysing the potential relationship between endocan-MVD and survival time, we found that patients with high endocan-MVD had a significantly poorer overall survival rate, which is consistent with the results for non-small-cell lung cancer and hepatocellular carcinoma[11,22]. With the Cox proportional hazards regression model, we found that endocan-MVD was an ideal marker to predict the prognosis in patients with gastric cancer.

The expression of endocan is regulated by a number of cytokines and growth factors, such as tumour necrosis factor-α, transforming growth factor-β1 and VEGF[23,24]. Among them, VEGF, as a major proangiogenic factor, has attracted the most attention. Recent studies have suggested that VEGF can induce the expression of endocan *in vitro*, and the VEGF-mediated induction of endocan mRNA was blocked by BIM (a PKC inhibitor) but not by PD98059 (an MEK1/2 inhibitor)[15]. However, endocan was reported less often in gastric cancer tissues and cells. In this study, we found that endocan-MVD was closely related to the expression of VEGF and VEGFR2 in gastric cancer tissues, which suggested that VEGF and VEGFR2 might induce the expression of endocan. The size of samples enrolled in our study was not large enough, and a subsequent study will employ larger samples based on a multicentre survey for further intervention. Despite this, analysing endocan-MVD might be useful in predicting prognoses and in choosing appropriate therapeutic modalities for patients with gastric cancer.

In conclusion, endocan had a high expression level in the endothelial cells of tumour vessels, and a significant association was found between endocan-MVD and the expression of VEGF or VEGFR2. The patients with high endocan-MVD had a significantly poorer overall survival rate than those with low endocan-MVD. This shows that endocan-MVD may play an important role in the processes of tumour therapy, which can be used as a critical factor for prognosis.

COMMENTS

Background

Endocan plays an extremely important role in the angiogenesis of tumours, and endocan-expressing microvessel density (MVD) (endocan-MVD) is likely to be valuable for the prognosis of malignancy. It has been reported that the secretion of endocan is strongly upregulated in the presence of vascular endothelial growth factor (VEGF). The exact relationship between endocan-MVD and the expression of VEGF and the value of endocan-MVD as a prognostic factor in patients with gastric cancer have not yet been defined.

Research frontiers

Vascular endocan, as a biomarker of neoangiogenesis, is found inside the vessels in different types of tumours, but not in gastric cancer. The exact relationship between endocan-MVD and the expression of VEGF in gastric cancer remains unclear. The research hotspot is to further clarify these issues.

Innovations and breakthroughs

Based on the investigation of clinical characteristics and endocan expression in 142 patients with gastric cancer, we first found that endocan-MVD had a positive correlation with VEGF and vascular endothelial growth factor receptor 2 (VEGFR2) in gastric cancer tissues. Patients with a high level of endocan-MVD had a significantly poorer overall survival rate than those with a low level of endocan-MVD.

Applications

The results of this study may clarify the role of endocan-MVD in the tumour neoangiogenesis in patients with gastric cancer, and provide strategies for the treatment of patients with advanced gastric cancer. Endocan-MVD may act as a valuable prognostic factor for survival in patients with gastric cancer.

Terminology

Endocan, previously called the endothelial cell specific molecule-1, which is a new member of the proteoglycan family, plays an extremely important role in the transformation, survival, proliferation, invasion, angiogenesis and metastasis of tumours. Microvascular density (MVD) is used as an objective evaluation index for tumour angiogenesis.

Peer-review

This is an interesting study about the expression of endocan in cancer vessels and the relationships between endocan and the expression of VEGF and prognosis in gastric cancer.

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ORIGINAL ARTICLE

Randomized Clinical Trial

Randomized study of lafutidine *vs* lansoprazole in patients with mild gastroesophageal reflux disease

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Abstract

AIM: To compare the clinical efficacy of the second-generation H2RA lafutidine with that of lansoprazole in Japanese patients with mild gastroesophageal reflux disease (GERD).

METHODS: Patients with symptoms of GERD and a diagnosis of grade A reflux esophagitis (according to the Los Angeles classification) were randomized to receive lafutidine (10 mg, twice daily) or lansoprazole (30 mg, once daily) for an initial 8 wk, followed by maintenance treatment comprising half-doses of the assigned drug for 24 wk. The primary endpoint was the frequency and severity of heartburn during initial and maintenance treatment. The secondary endpoints were the sum score of questions 2 and 3 in the Gastrointestinal Symptom Rating Scale (GSRS), and the satisfaction score.

RESULTS: Between April 2012 and March 2013, a total of 53 patients were enrolled, of whom 24 and 29 received lafutidine and lansoprazole, respectively. After 8 wk, the frequency and severity of heartburn was significantly reduced in both groups. However, lafutidine was significantly inferior to lansoprazole with regard to the severity of heartburn during initial and maintenance treatment (P = 0.016). The sum score of questions 2 and 3 in the GSRS, and satisfaction scores were also significantly worse in the lafutidine group than the lansoprazole group (P = 0.0068 and P = 0.0048, respectively).

CONCLUSION: The clinical efficacy of lafutidine was inferior to that of lansoprazole, even in Japanese patients with mild GERD.

Key words: Gastroesophageal reflux disease; Proton pump inhibitors; Histamine receptor-2 antagonists; Los Angeles classification

Core tip: The clinical efficacy of the second-generation H2RA lafutidine was inferior to that of lansoprazole, particularly during maintenance therapy, even in Japanese patients with mild gastroesophageal reflux disease.

Takenaka R, Okada H, Kawano S, Komazawa Y, Yoshinaga F, Nagata S, Inoue M, Komatsu H, Onogawa S, Kushiyama Y, Mukai S, Todo H, Okanobu H, Manabe N, Tanaka S, Haruma K, Kinoshita Y. Randomized study of lafutidine *vs* lansoprazole in patients with mild gastroesophageal reflux disease. *World J Gastroenterol* 2016; 22(23): 5430-5435 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i23/5430.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i23.5430

INTRODUCTION

Gastroesophageal reflux disease (GERD) is characterized by symptoms of heartburn and acid regurgitation. It is extremely common and has a chronic, relapsing disease course[1]. Standard pharmacological treatments for GERD comprise proton pump inhibitors (PPIs) and histamine receptor-2 antagonists (H2RAs), which act by suppressing gastric acid secretion. Of the two drug classes, PPIs are considered to be more cost-effective as initial treatment for GERD[2,3]. However, the cost-effectiveness of pharmacotherapy in Japanese patients with mild GERD has not been evaluated.

Lafutidine is a second-generation H2RA that has a potent and sustained anti-acid secretory effect. It acts by increasing plasma concentrations of calcitonin gene-related peptide and somatostatin, resulting in inhibition of postprandial acid secretion and gastroprotective activity[4]. In contrast to other H2RAs, the therapeutic action of lafutidine involves esophageal host-defense *via* capsaicin-sensitive afferent nerves[5]. The LAFORE trials conducted in Japanese patients with mild GERD (grade A in the Los Angeles classification) indicated that healing rates at 8 wk were 79.4% in the lafutidine groups and 68.3% in the famotidine group[6]. The cost of lafutidine treatment is 41.3 yen per day, and cheaper than half doses of PPIs (95.2 yen).

The aim of this study was to compare the clinical efficacy of lafutidine with that of lansoprazole as initial and maintenance treatment in Japanese patients with mild GERD.

MATERIALS AND METHODS

Study design

This was a phase Ⅲ, controlled study performed in 4

university hospitals and 11 of their affiliated hospitals in Japan between April 2012 and March 2013. The study was approved by the institutional review board of each participating hospital and was conducted in accordance to Good Clinical Practice guidelines. Written informed consent was obtained from all patients. The study is registered in the University Hospital Medical Network Clinical Trials Registry (unique trial number UMIN000006162).

Subjects

Inclusion criteria: Patients who were ⩾ 20 years old with symptoms of heartburn or regurgitation and a diagnosis of grade A reflux esophagitis according to the Los Angeles classification, as confirmed by endoscopic examination at least 1 wk prior to the observation period, were eligible for enrollment. Both the frequency and severity of symptoms were required to be ⩾ 3 on question 2 or 3 of the Gastrointestinal Symptom Rating Scale (GSRS).

Exclusion criteria: Patients with any of the following conditions were excluded: (1) gastric or duodenal ulcers (excluding ulcer scars); (2) esophageal, gastric or duodenal cancer; (3) the concurrent presence of Barrett's esophagus; (4) a history of upper gastrointestinal resection; (5) a history of receiving PPIs or H2RAs within the 2 wk prior to endoscopic examination; (6) comorbidity with severe cardiovascular, hepatic, or renal disease; (7) a history of allergy to lafutidine or lansoprazole; and (8) other conditions considered unsuitable for study participation by the attending physician.

Study methods

Eligible patients were randomized in a 1:1 ration to receive lafutidine (10 mg, twice daily) or lansoprazole (30 mg, once daily) for an initial 8 wk according to assignment lists generated by a permuted-block procedure. Patients were questioned on the frequency of heartburn during the week prior to initial treatment. After initial treatment, the frequency of heartburn was recorded daily for 2 wk. The number of episodes and the severity of heartburn were also assessed using the visual analog scale (VAS) and GSRS until 24 wk after the initial treatment-period. During initial treatment, concomitant administration of the following drugs was not permitted: (1) PPI; (2) H2RA; (3) prostaglandins; (4) mucosal protection drugs; (5) antacids; and (6) drugs that may affect upper gastrointestinal symptoms. Unless symptoms became worse, the allocated drug was administrated as initial treatment for 8 wk, followed by maintenance treatment for 24 wk. In the lafutidine group, a half dose was selected for maintenance treatment if symptoms had improved or disappeared at the first assessment, whereas the full dose was continued if symptoms had not improved. An asymptomatic state was defined as ⩽ 2 for both questions 2 and 3 on the GSRS. In the lansoprazole group, a half dose was administrated irrespective of symptoms, but was changed to the full dose if symptoms worsened. If symptoms had not improved at the first maintenance assessment, the attending physician was permitted to consider other treatment strategies. Patients underwent symptomatic evaluation every 8 wk for the duration of 32 wk. During the study, the number of episodes of heartburn was evaluated by reviewing patients' diaries. In addition to endoscopy, physical examinations and laboratory tests were performed to confirm the eligibility and safety of the patients.

Evaluation of symptoms

Patients' diaries were used to assess the frequency and severity of heartburn. The severity of heartburn and patient satisfaction were graded with VAS scores.

Endpoints

The primary endpoint was the frequency and severity of heart burn. The secondary endpoints were the sum score of questions 2 and 3 of the GSRS, and the patient satisfaction score, during initial and maintenance treatment.

Statistical analysis

Statistical differences between the two groups were determined with the chi-squared test or Fisher's exact test for discontinuous variables and the Mann-Whitney *U* test or Wilcoxon rank sum test for continuous variables. Repeated measures such as VAS and GSRS scores were analyzed by ANOVA. Statistical analyses were performed with the JMP (version 7) software package (SAS Institute, Cary, North Carolina, United States). *P* values ⩽ 0.05 were considered to denote statistically significant differences between groups. The statistical methods of this study were reviewed by Ryuta Takenaka from Tsuyama Chuo Hospital.

RESULTS

Patients flow

Between April 2012 and March 2013, the study enrolled 53 patients with heartburn or regurgitation who underwent upper gastrointestinal endoscopy. Among the 53 patients, 24 and 29 were randomized to the lafutidine and lansoprazole group, respectively. There were no significant differences between the two groups in background characteristics, except for gender distribution (Table 1).

During initial treatment, 8 patients discontinued the study, with the most common reasons being withdrawal of informed consent. Of the 45 patients assigned to receive maintenance treatment, 22 were excluded from the final analysis, primarily because of missing to follow-up (Figure 1).

Clinical outcomes during treatment

The frequency of heartburn according to the patients'

Table 1 Patients' clinical characteristics *n* (%)

	Lafutidine group (*n* = 24)	Lansoprazole group (*n* = 29)	*P* value
Age (median, range)	64 (27-80)	56 (26-85)	0.24
Gender (male/female)	8/16	22/7	0.0025
Hiatal hernia	13 (54)	19 (66)	0.57
H. pylori infection	2 (8.3)	3 (10.3)	> 0.99
Habits			
Alcohol	7 (29.2)	13 (44.8)	0.27
Tobacco	3 (12.5)	6 (20.7)	0.49
Coffee	14 (58.3)	15 (51.7)	0.78
Co-morbidity			
Hypertension	2 (8.3)	4 (13.8)	0.68
Diabetes mellitus	3 (12.5)	3 (10.3)	> 0.99
Cerebrovascular disease	0 (0)	0 (0)	-
Cardiovascular disease	0 (0)	0 (0)	-
Liver disease	1 (4.2)	3 (10.3)	0.62
Renal failure	0 (0)	0 (0)	-
Malignancy	2 (8.3)	2 (6.9)	> 0.99

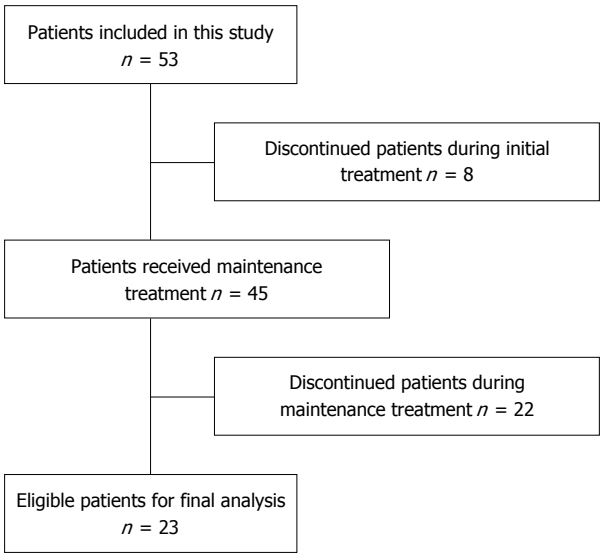

Figure 1 Patient flow.

questionnaires was lower after initial treatment in both groups (Figure 2). However, the change in heartburn frequency was not statistically significantly different in either group (Figure 3). The severity of heartburn, evaluated using VAS, was also significantly reduced after initial treatment in both groups. Worsening of symptom during maintenance treatment did not occur in either group. However, lafutidine was significantly inferior to lansoprazole in reducing heartburn severity (Figure 4). Similarly, the sum score of questions 2 and 3 in the GSRS and the satisfaction score were both significantly worse in the lafutidine group than those in the lansoprazole group, particularly during maintenance therapy (Figures 5 and 6, respectively).

DISCUSSION

GERD is a chronic disorder and long-term acid-suppression therapy is necessary in most cases[7].

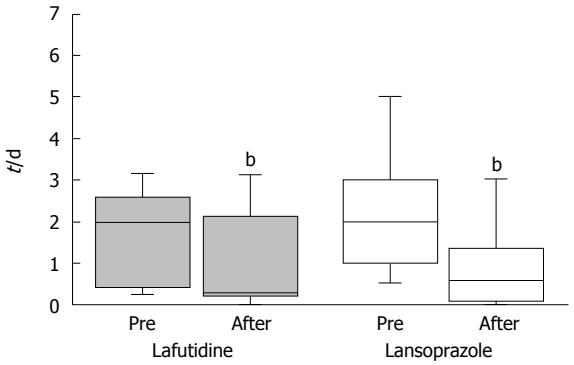

Figure 2 Frequency of heartburn evaluated using patient' questionnaires. Data are shown as a boxplot. In both groups, the frequency of heartburn was reduced after initial treatment, $^{b}P < 0.01$.

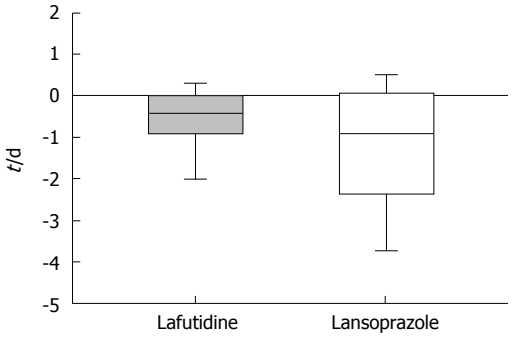

Figure 3 Change in heartburn frequency after initial treatment. Data are shown as a boxplot. There were no significant difference between the lafutidine group and the lansoprazole group ($P = 0.26$).

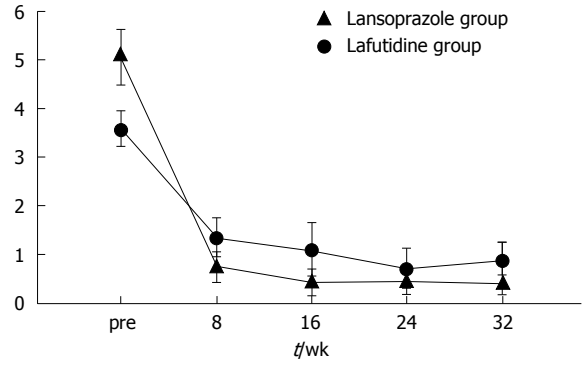

Figure 4 Severity of heartburn evaluated using visual analog scale scores. Data are expressed as means ± SE. VAS scores in the lafutidine group were significantly worse than in the lansoprazole group ($P = 0.016$, ANOVA for repeated measures). VAS: Visual analog scale.

A meta-analysis of 34 trials that included 1,314 individuals demonstrated that PPIs were significantly more effective than H2RAs in relieving heartburn in GERD (RR = 0.66, 95%CI: 0.60-0.73) as well as in patients with non-erosive reflux disease on upper endoscopy (RR = 0.78, 95%CI: 0.62-0.97)[8]. Because no reports have been published concerning the cost-effectiveness of pharmacotherapy in Japanese patients with mild GERD, which is a common condition in Japan, the present study was designed to evaluate

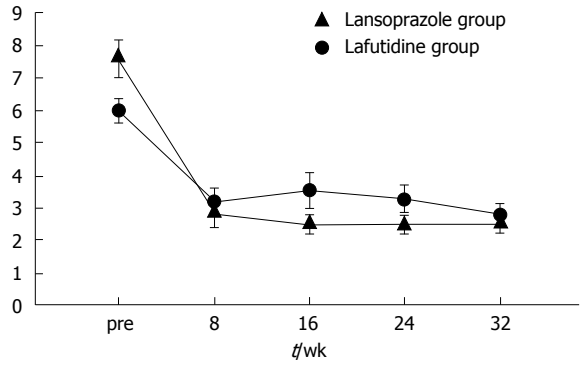

Figure 5 Sum score of question 2 and question 3 in the GSRS score. Data are expressed as means ± SE. VAS scores in the lafutidine group were significantly worse than in the lansoprazole group (P = 0.0068, ANOVA for repeated measures). VAS: Visual analog scale.

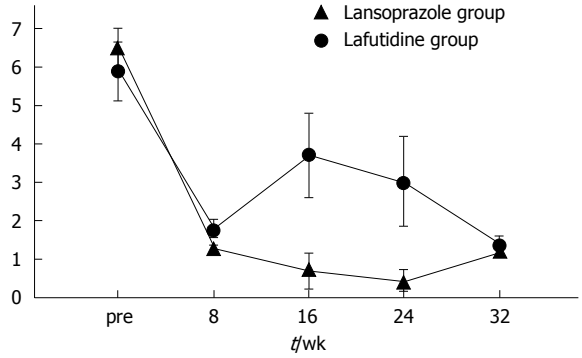

Figure 6 Satisfaction score evaluated using visual analog scale scores. Data are expressed as means ± SE. VAS scores in the lafutidine group were significantly worse than the lansoprazole group (P = 0.0048, ANOVA for repeated measures). VAS: Visual analog scale.

the efficacy of the second-generation H2RA lafutidine. However, lansoprazole was superior to lafutidine in Japanese patients with mild GERD, not only with respect to lowering the severity of heartburn, but also the satisfaction score. The GSRS score was also better in the lansoprazole than the lafutidine group. Thus, the hypothesis that lafutidine has similar efficacy and superior cost-effectiveness compared with lansoprazole in Japanese patients with mild GERD was not confirmed.

Limitations of PPIs include a higher cost than H2RAs, and potential side effects such as hypochlorhydria and hypergastrinemia. In particular, PPI-related hypochlorhydria is concern because it may increase susceptibility to infections, for example Clostridium difficile-associated diarrhea[9-11], as well as malabsorption leading to hypomagnesemia[12]. Hypochlorhydria may theoretically reduce calcium absorption[13]. A meta-analysis that included 11 cohort and case-control studies examined the risk of fractures associated with PPI use, and showed that the risk of hip fracture, spine fracture, and any-site fracture was increased among PPI users compared with nonusers (RR = 1.30, 95%CI: 1.19-1.43; RR = 1.56, 95%CI: 1.31-1.85; and RR = 1.16, 95%CI: 1.02-1.32, respectively)[14]. Therefore, once an asymptomatic state has been attained, tapering or cessation of medication should be considered in patients with GERD.

In the present study, initial therapy improved clinical outcomes such as heartburn and patient satisfaction score in both the lafutidine group and the lansoprazole group. However, the satisfaction score and GSRS score became worse during maintenance therapy in the lafutidine group. The effect of acid suppression in the lafutidine group was weak during the maintenance therapy. Two possible mechanisms may explain this observation. Firstly, patients may develop tolerance to lafutidine during maintenance therapy. As previously reported in a study assessing ranitidine and famotidine, the development of tachyphylaxis within 2 to 6 wk of initiation of H2RAs limits their use as maintenance therapy for GERD[15]. Secondly, the dose of lafutidine during maintenance therapy was low for symptomatic relief. If a 10-mg dose of lafutidine was administered twice daily during maintenance therapy, symptomatic severity may have been similar to that in the lansoprazole group. The costs of 10 mg of lafutidine twice daily and a half dose of lansoprazole were 82.6 yen and 95.2 yen, respectively. Therefore, future studies are planned to evaluate 10 mg of lafutidine twice daily for maintenance therapy, with the aim of proving our hypothesis that lafutidine has similar efficacy and superior cost-performance to lansoprazole in Japanese patients with mild GERD.

This study has some limitations. The sample size was relatively small. Furthermore, a substantial number of patients discontinued the study although the proportion of patients lost to follow-up was similar in the two treatment groups. In addition, the male/female ratio was significantly smaller in the lafutidine than the lansoprazole group, which may have had an influence on clinical outcomes.

In conclusion, the efficacy of a second-generation H2RA over a PPI in Japanese patients with mild GERD was not demonstrated, most notably during maintenance therapy.

COMMENTS

Background

Proton pump inhibitors (PPIs) are considered to be more cost-effective for patients with gastroesophageal reflux disease (GERD) than first-generation histamine receptor-2 antagonists (H2RAs). Lafutidine is a second-generation H2RA that has a potent and sustained anti-acid secretory effect. In LAFORE trials conducted in Japanese patients with mild GERD, lafutidine was superior to first-generation H2RA (famotidine). The aim of this study was to compare the clinical efficacy of lafutidine with that of PPI (lansoprazole) as initial and maintenance treatment in Japanese patients with mild GERD.

Research frontiers

GERD is a chronic disorder and long-term acid-suppression therapy is necessary in most cases. Limitations of PPIs include a higher cost than H2RAs, and potential side effects related to hypochlorhydria and hypergastrinemia.

Innovations and breakthroughs

Lansoprazole was superior to lafutidine in Japanese patients with mild GERD, not only with respect to lowering the severity of heartburn, but also the satisfaction score. The hypothesis that lafutidine had similar efficacy and superior cost-effectiveness compared with lansoprazole in Japanese patients with mild GERD was not confirmed.

Applications

The efficacy of a second-generation H2RA over a PPI in Japanese patients with mild GERD was not demonstrated, most notably during maintenance therapy.

Terminology

Lafutidine is a second-generation H2RA and shown to be superior to first-generation H2RA (famotidine).

Peer-review

Interesting and well written study.

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META-ANALYSIS

Efficacy and adverse events of cold *vs* hot polypectomy: A meta-analysis

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Abstract

AIM: To compare previously reported randomized controlled studies (RCTs) of cold and hot polypectomy, we systematically reviewed and clarify the utility of cold polypectomy over hot with respect to efficacy and adverse events.

METHODS: A meta-analysis was conducted to evaluate the predominance of cold and hot polypectomy for removing colon polyps. Published articles and abstracts from worldwide conferences were searched using the keywords "cold polypectomy". RCTs that compared either or both the effects or adverse events of cold polypectomy with those of hot polypectomy were collected. The patients' demographics, endoscopic procedures, No. of examined lesions, lesion size, macroscopic and histologic findings, rates of incomplete resection, bleeding amount, perforation, and length of procedure were extracted from each study. A forest plot analysis was used to verify the relative strength of the effects and adverse events of each procedure. A funnel plot was generated to assess the possibility of publication bias.

RESULTS: Ultimately, six RCTs were selected. No significant differences were noted in the average lesion size (less than 10 mm) between the cold and hot polypectomy groups in each study. Further, the rates of complete resection and adverse events, including

delayed bleeding, did not differ markedly between cold and hot polypectomy. The average procedural time in the cold polypectomy group was significantly shorter than in the hot polypectomy group.

CONCLUSION: Cold polypectomy is a time-saving procedure for removing small polyps with markedly similar curability and safety to hot polypectomy.

Key words: Cold polypectomy; Hot polypectomy; Colon adenoma; Conventional polypectomy; Colon neoplasm; Endoscopic mucosal resection; Bleeding

Core tip: We conducted a meta-analysis to evaluate the predominance of cold and hot polypectomy for removing colon polyps. The patients' demographics, endoscopic procedures, No. of examined lesions, lesion size, macroscopic and histologic findings, rates of incomplete resection, bleeding, and perforation, and length of procedure were extracted from six randomized controlled studies. The rates of complete resection and adverse events did not markedly differ between cold and hot polypectomy. However, the procedural time was significantly shorter in the cold polypectomy group. These results suggest that cold polypectomy is a time-saving procedure for removing small polyps with similar curability and safety to hot polypectomy.

Fujiya M, Sato H, Ueno N, Sakatani A, Tanaka K, Dokoshi T, Fujibayashi S, Nomura Y, Kashima S, Gotoh T, Sasajima J, Moriichi K, Watari J, Kohgo Y. Efficacy and adverse events of cold *vs* hot polypectomy: A meta-analysis. *World J Gastroenterol* 2016; 22(23): 5436-5444 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i23/5436.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i23.5436

INTRODUCTION

Colon cancer is an extremely common cancer with high mortality in both Asia and the West. Because colon cancer develops due to an accumulation of genetic alterations prompting the transformation of the normal colon epithelium to adenoma and subsequently adenocarcinoma[1,2], removal of colon adenomas is considered crucial for preventing the development of cancer[3]. Indeed, several trials have demonstrated that removal of colon adenoma successfully decreases the mortality rate of colon cancer[3,4].

Polypectomy is a minimally invasive and easy-to-learn procedure for removing colon adenomas, particularly elevated-shaped ones. The polypectomy procedure for colon neoplasms generally uses a snare to enclose the lesion, which is then cut using a high-frequency generator. This procedure is known as hot polypectomy. Cutting and coagulation of the lesion with a high-frequency generator is generally believed to help prevent bleeding after polypectomy, although this procedure can occasionally cause perforation after lesion removal due to the burning of the intestinal wall.

An alternative procedure, known as cold polypectomy, simply removes lesions using a snare with no high-frequency generator and is believed to be as effective as hot polypectomy for removing colon neoplasms, but with lower risk of complications, such as perforation. In their examination of 210 constitutive patients after cold polypectomy for polyps 5 mm or less in size, Tappero *et al*[5] observed no adverse effects, including immediate or delayed bleeding or perforation. In addition, several uncontrolled studies have further confirmed the safety of cold polypectomy for removing small polyps[5-8]. However, 2 uncontrolled studies reported incomplete resection rates of 5% and 11% for cold polypectomy[9,10], which could still allow for progression to colon cancer. Any advantages of cold polypectomy over hot with respect to efficacy and adverse events therefore remain unclear.

To clarify the utility of cold polypectomy concerning efficacy and adverse events, we systematically reviewed previously-reported randomized controlled studies (RCTs) comparing cold and hot polypectomy procedures.

MATERIALS AND METHODS

Retrieval strategy and quality assessment

Articles posted on PubMed (as of March 2015) and abstracts of worldwide conferences described in *Gastrointestinal Endoscopy and Gut* were searched using the keywords "cold polypectomy". The language was limited to studies published in English.

The results were reviewed by two evaluators. The abstracts were not blinded for authors, institutions, or journals during review. All studies comparing the effects and adverse events of cold polypectomy with those of hot polypectomy were collected, regardless of whether or not the data were part of the primary or secondary endpoint. When multiple articles were published from the same institution or study group, the most recent one was selected for our analysis. Even if the study group was included in multiple articles, the data were applied when the study population was different in each study. Reviews, case reports, abstracts, and presentations from meetings were excluded.

The risk of bias was evaluated in accordance with the Cochrane Handbook for Systematic Reviews of Interventions using the following parameters: adequacy of random sequence generation; allocation concealment; blinding of the participants, personnel and outcome assessors; incomplete outcome data and selective outcome reporting. The Jadad score was used to evaluate the quality of each study[11]. Briefly, the details of the randomization and blinding procedures

Table 1 Quality assessment of bias in included studies

Ref.	Risk of bias						Jadad score			
	Adequacy of random sequence generation	Allocation concealment	Blinding of the participants, personnel and outcome assessors	Blind outcome assessment	Incomplete outcome data	Selective outcome reporting	Details of randomization	Blinding procedures	Information regarding withdrawals	Total score
Horiuchi *et al*[15], 2010	High	High	Unclear	Unclear	Unclear	Low	1	1	0	2
Ichise *et al*[16], 2011	Unclear	Low	Unclear	Unclear	Unclear	Low	2	1	0	3
Paspatis *et al*[17], 2011	Low	Unclear	Unclear	Unclear	Unclear	Low	2	0	1	3
Aslan *et al*[18], 2013	High	High	Unclear	Unclear	High	Unclear	1	1	0	2
Horiuchi *et al*[21], 2013	Low	Low	Unclear	Unclear	Unclear	Unclear	2	2	0	4
Gomez *et al*[20], 2014	Low	Low	Unclear	Unclear	Low	Unclear	2	1	1	3

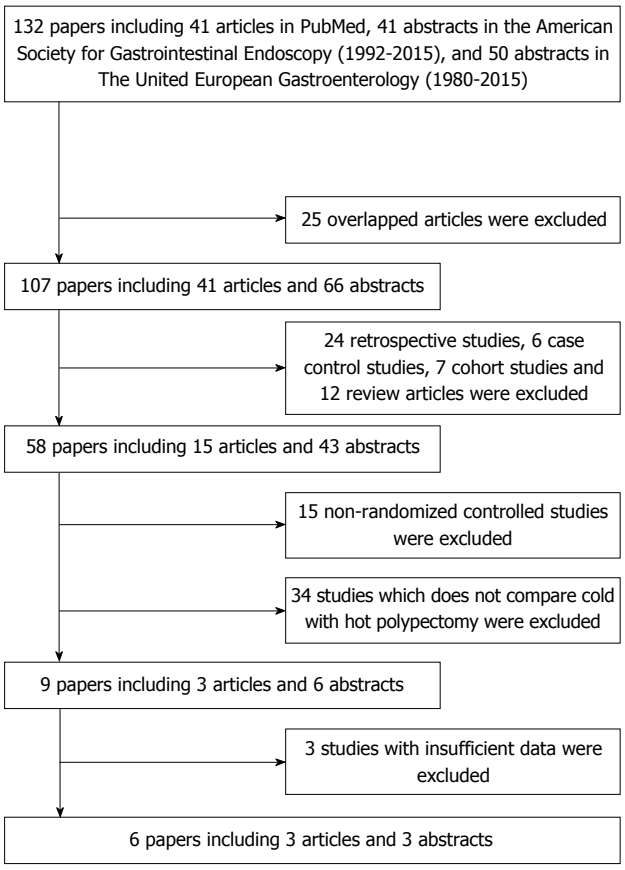

Figure 1 PRISMA flow diagram.

and information regarding withdrawals were first evaluated. One or two points were then awarded for specifying the randomization and blinding procedures and one point for a statement of information regarding withdrawals (Table 1). A funnel plot was generated to assess the possibility of any publication bias[12].

Data collection and bias assessment

The patients' demographics, endoscopic procedures, No. of examined lesions, lesion size, macroscopic and histologic findings, rates of incomplete resection, occurrence of immediate or delayed bleeding and perforation, and procedure duration were extracted from each study. The macroscopic and histologic findings were assessed based on Paris classification[13] and Vienna classification[14], respectively. Incomplete resection was defined as the clear presence of tumor cells in the margin of the removed specimen on histologic examination. The procedure duration was defined as the total time elapsed during the procedure. A forest plot analysis was used to verify the relative strength of the effects and adverse events of each procedure in multiple quantitative scientific studies. The between-study heterogeneity was tested and quantified using the Cochran Q statistic and the I^2 statistic, respectively.

Statistical analysis

The relative risk (RR) and 95% confidence interval (CI) were calculated for each variable based on the data. Statistical analyses were performed using the Cochrane Collaboration's Revman 5.0 software program (The Cochrane Collaboration, Oxford, Oxfordshire, United Kingdom). The inter-study heterogeneity was assessed using the Cochran Q test, with the significance level set at $P = 0.1$, and was quantified using the I^2 statistic. If any obvious inter-study heterogeneity was noted ($I^2 > 50\%$), the random effects model was chosen; otherwise, the fixed effects model was chosen.

RESULTS

Evaluation and details of the selected studies

A total of 131 reports were initially retrieved (Figure 1). After screening the titles, abstracts, or full text and excluding reviews, case reports, uncontrolled tests, and basic research studies, seven studies were selected[15-21]. Of these seven, one study was

Table 2 Details of included studies

Ref.	Patient, Age, mean ± SD or median (range)	Gender (M/F)	No. of polyps	Tumor size, mean ± SD (mm)	Endoscopic procedure for cold polypectomy	No. of all adverse events	Macroscopic appearances		Histological findings			Complete retrieval rate	Post-polypectomy bleeding	Procedure time, mean ± SD (min)
							0-Ⅰ	0-Ⅱ	High grade adenoma	Low grade adenoma	Hyperplastic polyp			
Cold polypectomy														
Horiuchi *et al*[15], 2010	NA	NA	94	NA	Snare	1	NA	NA	NA	NA	NA	96	0	18
Ichise *et al*[16], 2011	65.1 ± 11	25/15	101	5.7 ± 4	Snare	1	89	12	1	94	6	96	0	18 ± 6
Paspatis *et al*[17], 2011	59.4 ± 13.6	107/101	636	5.3 ± 1.4	Snare	19	NA	NA	NA	NA	NA	96	0	23.3 ± 4.8
Aslan *et al*[18], 2013	59.5 ± 14.9	32/17	78	7.21 ± 1.4	Snare	1	NA	NA	NA	NA	NA	94.9	1	NA
Horiuchi *et al*[21], 2013	67.0 ± 13	25/10	78	6.5 ± 1.2	Snare	4	55	23	2	70	6	94	0	16 ± 7
Gomez *et al*[20], 2014	NA	NA	44	NA	Snare or Forceps	0	NA	NA	NA	NA	NA	90 (cold snare), 89 (cold forceps)	0	NA
Hot polypectomy														
Horiuchi *et al*[15], 2010	NA	NA	92	NA	-	8	NA	NA	NA	NA	NA	97	0	25
Ichise *et al*[16], 2011	65.5 ± 12	28/12	104	5.5 ± 6	-	8	95	9	1	93	10	96	0	25 ± 7
Paspatis *et al*[17], 2011	61.3 ± 11	125/81	619	5.67 ± 1.3	-	2	NA	NA	NA	NA	NA	96	0	29.6 ± 7.4
Aslan *et al*[18], 2013	58.3 ± 13.5	36/12	71	7.56 ± 1.45	-	1	NA	NA	NA	NA	NA	94.4	1	NA
Horiuchi *et al*[21], 2013	67.3 ± 12	24/11	81	6.8 ± 1.3	-	16	62	19	2	72	7	93	5	26 ± 9
Gomez *et al*[20], 2014	NA	NA	18	NA	-	0	NA	NA	NA	NA	NA	94	0	NA

NA: Data not available.

excluded because some of the enrolled cases were believed to overlap with those of other reports[21]. The risk of bias was then assessed for the remaining six studies in accordance with the Cochrane Handbook for Systematic Reviews of Interventions (Table 1).

All six included studies were published from 2010 to 2014 (Table 2). The mean or median age of patients treated with cold polypectomy ranged from 59.4 to 67.0 years old, while those of patients treated with hot polypectomy ranged from 58.3 to 67.3 years old, which were described in 4 studies. The ratio of males to females in all described cases was 1.06 to 2.50 among cold polypectomy cases and 1.54 to 3.00 among hot polypectomy cases. Cold polypectomy with a snare only was used in 5 studies, while both snare and forceps were used in 1 study. The macroscopic appearances of the lesions in 2 studies were 0-I in 89 and 55 lesions and 0-Ⅱ in 12 and 23 lesions among cold polypectomy cases, and 0-I in 95 and 62 lesions and 0-Ⅱ in 9 and 19 lesions among hot polypectomy cases. The histologic diagnoses of the lesions in these 2 studies were 187 and 142 low-grade adenomas, 2 and 4 high-grade adenomas, and 16 and 13 hyperplastic polyps.

Tumor size

Two and four studies investigated lesions measuring 10 mm or less[18,19] and 8 mm or less in diameter[15-17,20], respectively, indicating that all studies investigated the effect of cold and hot polypectomy on the treatment of small polyps. Four studies described the average size and standard deviation (SD) of the examined lesions[16-19], and no significant difference was noted in the average size (less than 10 mm) between the cold and hot polypectomy groups in each study (Table 2).

Incomplete resection

Three studies described the rate of curative resection[16,17,19]. These reports included a total of 815 lesions removed by cold polypectomy and 804 removed by hot polypectomy. Total rates of curative resection by cold and hot polypectomy were 98.5% and 97.8%, respectively. Because the between-study heterogeneity of these 3 studies was low (P = 0.5134, I^2 = 0%), a fixed-effects model was used to analyze the rate of curative resection. The RR for all lesions was 0.68, and the 95%CI ranged from 0.33 to 1.38 (Figure 2A), findings which indicate that the rate of complete resection did not markedly differ between cold and hot polypectomy groups. Evaluation

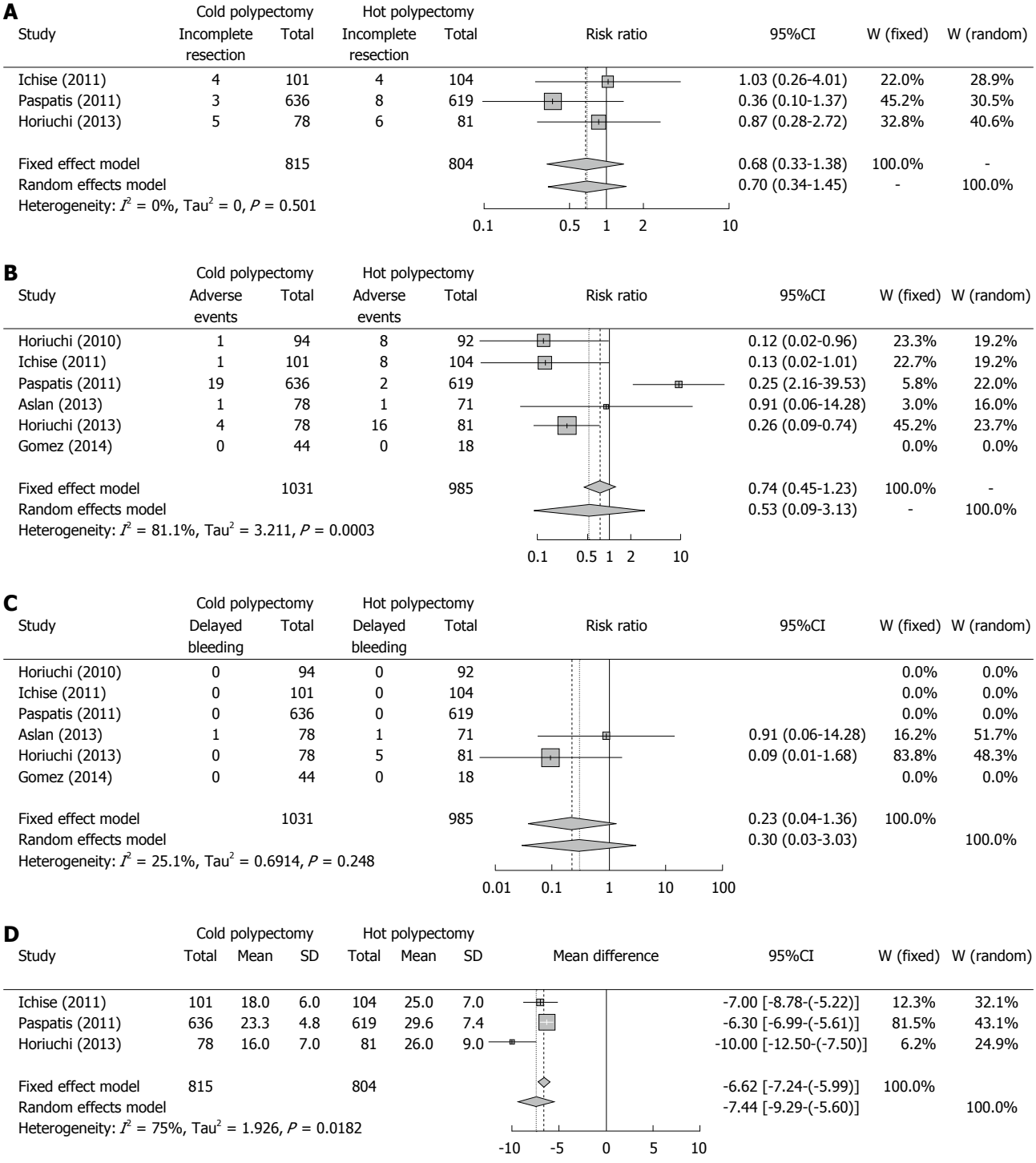

Figure 2 Forest plots were used to verify the relative strength of the diagnostic value of each procedure. The results regarding the rates of incomplete resection (A), adverse events (B), delayed bleeding (C), and procedure duration (D) are shown. ESD: Endoscopic submucosal dissection; MD: Mean difference; SD: Standard deviation; CI: Confidence interval; W: Weight; OR: Odds ratio.

of publication bias was difficult due to the small No. of studies, as shown in the funnel plot (Figure 3A).

Adverse events

All six studies reported the rate of adverse events, including bleeding and abdominal pain or discomfort[15-20]. These six reports included a total of 1031 lesions removed by cold polypectomy and 985 removed by hot polypectomy. Total rates of adverse events by cold and hot polypectomy were 2.5% and 3.6%, respectively. Because the between-study heterogeneity of these six studies was high (P = 0.0002, I^2 = 81.8%), a random-effects model was used to analyze the rate of adverse events. The RR for all lesions was 0.53, and the 95%CI ranged from 0.09 to 3.13 (Figure 2B), findings which indicated that the rate of adverse events did not

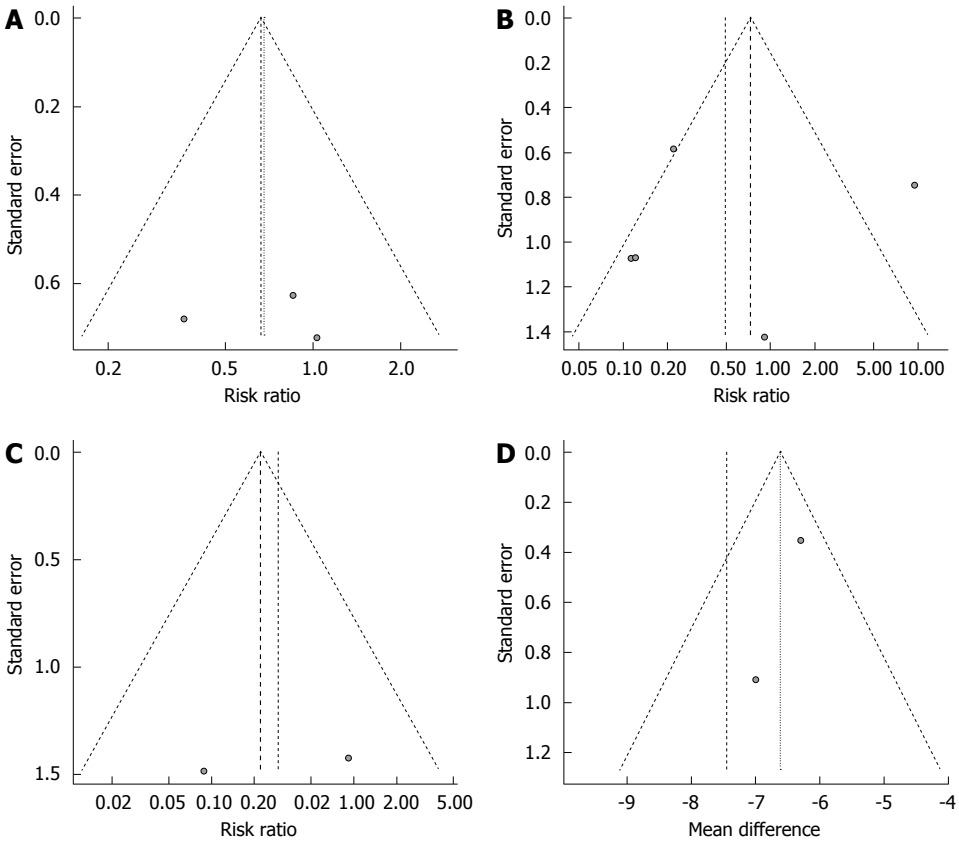

Figure 3 Funnel plots were used to clarify any publication bias. The results regarding the rates of incomplete resection (A), adverse events (B), delayed bleeding (C), and procedure duration (D) are shown.

markedly differ between cold and hot polypectomy procedures. An evaluation of publication bias was difficult to carry out due to the small No. of studies, as shown in the funnel plot (Figure 3B).

Delayed bleeding was observed in only two studies, with conflicting findings in both[18,19]. While Aslan *et al*[18] noted no marked differences in the rate of bleeding between cold and hot polypectomy groups, Horiuchi *et al*[19] observed a higher bleeding rate in the hot polypectomy group than in the cold procedure group among patients given anticoagulants. Total rates of delayed bleeding by cold and hot polypectomy were 0.1% and 0.6%, respectively. Because the between-study heterogeneity of these six studies was high (P = 0.2427, I^2 = 26.7%), a fixed-effects model was used to analyze the rate of bleeding. The RR for all lesions was 0.23, and the 95%CI ranged from 0.04 to 1.36 (Figure 2C). An evaluation of publication bias was difficult to carry out due to the small No. of studies, as shown in the funnel plot (Figure 3C).

Perforation

No perforation occurred in any of the six studies investigated.

Procedure duration

Three studies described the average and SD of procedure duration[16,17,19]. The average durations for these three studies ranged from 16.0 to 23.3 min in the cold polypectomy group and from 25.0 to 29.6 min in the hot polypectomy group. The cold polypectomy procedures were significantly shorter in duration than those using hot polypectomy in all three studies. Because the between-study heterogeneity of these three studies was high (P = 0.0182, I^2 = 75.0%), a random-effects model was used to analyze the length of the procedure. The mean difference for all lesions was -7.44, and the 95%CI ranged from -9.29 to -5.60 (Figure 2D), findings which indicate that the cold polypectomy procedure was shorter than the hot polypectomy procedure. An evaluation of publication bias was difficult to carry out due to the small No. of studies, as shown in the funnel plot (Figure 3D).

DISCUSSION

To our knowledge, the present systematic review is the first meta-analysis to compare the efficacy and safety of cold polypectomy for removing colon tumors with those of hot polypectomy procedures based on previously published RCTs. In our analysis, the rates of incomplete resection and adverse events in the cold polypectomy group were not significantly different from those in the hot polypectomy group, suggesting that cold polypectomy possesses the same efficacy and safety as hot polypectomy. Further, the procedural

time was significantly shorter in the cold polypectomy group, suggesting that cold polypectomy is also a time-saving procedure compared to hot polypectomy.

The present analysis also investigated the sizes of polyps encountered in the six RCTs and confirmed a lack of any marked difference in size between the two polypectomy groups, which indicates low bias associated with tumor size. However, all polyps encountered in these RCTs were 10 mm or less in diameter, and most were 8 mm or less. The findings from the present analysis therefore apply only with respect to removal of small polyps, and the risks of incomplete resection and adverse events with cold polypectomy for removing large polyps remains unclear. Previous studies have reported that rates of incomplete resection and adverse events of endoscopic resection are proportional to polyp size[22,23]. Recently, Tribonias *et al*[24] developed a new method for removing flat polyps measuring larger than 10 mm in size using a pulling technique and proposed the potential utility of cold polypectomy in removing large polyps. Further analysis of studies involving patients with large polyps using novel methods will be needed to determine whether or not cold polypectomy is a practical option for removing polyps larger than 10 mm in size.

Cold polypectomy can be performed *via* two methods: cold snare polypectomy and cold forceps polypectomy. The cold snare procedure uses snares to encircle and cut polyps, while the cold forceps procedure uses large forceps to "bite" polyps. While cold forceps polypectomy is easier to perform than cold snare polypectomy, the rate of incomplete resection is believed to be higher for cold forceps polypectomy[25], coming in at approximately 20% even when using jumbo-sized forceps[26]. In the RCTs retrieved in the present meta-analysis, only one study used the cold forceps procedure (and only for a portion of cases)[20], while other five studies used only the cold snare procedure[15-19]. Because the RCT using the cold forceps procedure did not describe the rate of incomplete resection, our meta-analysis of the rate of incomplete resection was not influenced by any bias related to cold polypectomy procedure.

Lee *et al*[27] showed in their RCT that the rate of incomplete resection for cold snare polypectomy was significantly lower than that in the cold forceps polypectomy group (93.2% *vs* 75.9%). Similarly, Kim *et al*[28] showed in their RCT that the rate of incomplete resection for cold snare polypectomy removing 5- to 7-mm-sized adenomatous polyps was significantly lower than that for cold forceps polypectomy (93.8% *vs* 70.3%). These findings suggest that the cold snare procedure is indeed superior to the cold forceps procedure for removing colon polyps.

Several limitations to the present meta-analysis warrant mention. First, the rates of recurrence after cold and hot polypectomies were not investigated, although histological margins were assessed. Because histological findings do not always predict future recurrence, a true rate of incomplete resection should be assessed through follow-up studies. Second, the difference in the mortality rates of colon cancer in populations with and without cold polypectomy was not investigated. Because the goal of polypectomy for removing colon adenoma is to reduce risk of colon cancer-associated death, the efficacy of cold polypectomy should be assessed based on improvement in colon cancer-related mortality. Long-term follow-up studies are therefore needed to clarify whether or not cold polypectomy does indeed reduce the mortality of colon cancer. Third, the skill and experience of each physician is believed to be another source of bias affecting the rates of incomplete resection and adverse events as well as the procedural time, which should be resolved by future studies.

In conclusion, we conducted the first meta-analysis concerning the efficacy and adverse events of cold polypectomy in comparison to hot polypectomy. Our findings demonstrate that cold polypectomy possesses similar curability and safety to hot polypectomy. Further, cold polypectomy is a time-saving procedure compared to hot polypectomy and is thus recommended for use over the hot procedure in removing small polyps. However, further analyses are needed to assess the feasibility of cold polypectomy for removing large polyps as well as to evaluate any bias associated with the skill and experience of each physician and the type of endoscopic device used.

COMMENTS

Background

Interest in cold polypectomy for removing small polyps is growing among endoscopists worldwide. In several prospective randomized trials, cold polypectomy has been proven effective in removing colon polyps less than 10 mm in diameter. However, no systematic review concerning the efficacy and safety of cold polypectomy, versus conventional hot polypectomy, has been published.

Research frontiers

Polypectomy is an easy-to-learn, minimally invasive procedure for removing colon polyps. Cold polypectomy is believed to be a safer procedure with fewer complications than hot polypectomy.

Innovations and breakthroughs

To confirm the safety and efficacy of cold polypectomy for removing colon polyps, the authors evaluated the findings from six prospective randomized controlled trials (RCTs). This is the first meta-analysis of RCTs comparing cold and hot polypectomy outcomes.

Applications

The present findings support the safety and efficacy of cold polypectomy for removing colon polyps less than 10 mm in diameter.

Terminology

Cochrane Handbook for Systematic Reviews of Interventions: This book is an official guide describing in detail the process of preparing and maintaining Cochrane systematic reviews on the effects of healthcare interventions. Adequacy of random sequence generation: A simple statement such as 'we randomly allocated' or 'using a randomized design' is often insufficient to prove that the allocation sequence was genuinely randomized. Authors commonly use

the term 'randomized' even when it is not justified, and many trials with declared systematic allocation are described by the authors as being randomized. When in doubt, the adequacy of sequence generation should be considered unclear. Allocation concealment: Proper allocation sequence concealment secures strict implementation of an allocation sequence without foreknowledge of intervention assignments. Methods for allocation concealment refer to techniques used to implement the sequence, not to generate it. However, most allocation sequences that are deemed inadequate, such as allocation based on day of admission or case record number, cannot be adequately concealed, and so fail on both counts. It is theoretically possible, yet unlikely, that an inadequate sequence is adequately concealed. However, it is not uncommon for an adequate allocation sequence to be inadequately concealed, such as if the sequence is posted on a staff room wall. Blinding of the participants, personnel, and outcome assessors: Study reports often describe blinding in broad terms, such as 'double-blinded'. This term makes it impossible to know who was blinded. Such terms are often used inconsistently, and the frequency of explicit reporting of the blinding status of study participants and personnel remains low. A review of methods used for blinding highlights the variety of methods used in practice. The following may help review authors assess whether or not any blinding of participants and personnel in a study was likely to be sufficient to protect against bias when using the Collaboration's tool: When considering the risk of bias from lack of blinding of participants and personnel, consider specifically (1) who was and was not blinded; and (2) risk of bias in actual outcomes due to lack of blinding during the study. Incomplete outcome data and selective outcome reporting: The risk of bias arising from incomplete outcome data depends on several factors, including the amount and distribution across intervention groups, the reasons for outcomes being missing, the likely difference in outcome between participants with and without data, what study authors have done to address the problem in their reported analyses, and the clinical context. Therefore, it is not possible to formulate a simple rule for judging a study to be at low or high risk of bias. The following may help review authors assess whether or not incomplete outcome data can be addressed in a way that protects against bias when using the Collaboration's tool. Jadad score: The Jadad score assesses the quality of published clinical trials based on methods relevant to random assignment, double blinding, and the flow of patients. The score is assigned based on four items. Forest plot: A forest plot is a graphic display of estimated results from a No. of scientific studies addressing the same question, along with the overall results. This plot was developed for use in medical research as a means of graphically representing a meta-analysis of the results of RCTs. Funnel plot: A funnel plot is a graph designed to check for the existence of publication bias; funnel plots are commonly used in systematic reviews and meta-analyses.

Peer-review

This systematic review and meta-analysis of six RCTs provides valuable information supporting cold polypectomy as a safe and time-saving procedure for removing small polyps with no risk of additional complications.

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META-ANALYSIS

Nonbismuth concomitant quadruple therapy for *Helicobacter pylori* eradication in Chinese regions: A meta-analysis of randomized controlled trials

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Abstract

AIM: To evaluate the applicability of nonbismuth concomitant quadruple therapy for *Helicobacter pylori* (*H. pylori*) eradication in Chinese regions.

METHODS: A systematic review and meta-analysis of randomized controlled trials was performed to evaluate the efficacy of nonbismuth concomitant quadruple therapy between sequential therapy or triple therapy for *H. pylori* eradication in Chinese regions. The defined Chinese regions include China, Hong Kong, Taiwan, and Singapore. The primary outcome was the *H. pylori* eradication rate; the secondary outcome was the compliance with therapy. The PubMed, Embase, Scopus, and Cochrane databases were searched for studies published in the period up to March 2016 with no language restriction.

RESULTS: We reviewed six randomized controlled trials and 1616 patients. In 3 trials comparing concomitant quadruple therapy with triple therapy, the *H. pylori* eradication rate was significantly higher for 7-d

nonbismuth concomitant quadruple therapy than for 7-d triple therapy (91.2% *vs* 77.9%, risk ratio = 1.17, 95%CI: 1.09-1.25). In 3 trials comparing quadruple therapy with sequential therapy, the eradication rate was not significant between groups (86.9% *vs* 86.0%). However, higher compliance was achieved with concomitant therapy than with sequential therapy.

CONCLUSION: The *H. pylori* eradication rate was higher for nonbismuth concomitant quadruple therapy than for triple therapy. Moreover, higher compliance was achieved with nonbismuth concomitant quadruple therapy than with sequential therapy. Thus, nonbismuth concomitant quadruple therapy should be the first-line treatment in Chinese regions.

Key words: *Helicobacter pylori* eradication; Nonbismuth concomitant quadruple therapy; Peptic ulcer; Chinese region

Core tip: *Helicobacter pylori* (*H. pylori*) infection is highly prevalent in Chinese regions and associated with peptic ulcers. Currently, triple and sequential therapies have been widely used to eradicate *H. pylori*. Nonbismuth concomitant quadruple therapy is an alternative treatment with high efficacy. Our meta-analysis revealed that a higher *H. pylori* eradication rate was achieved with 7-d concomitant therapy than with 7-d triple therapy. The eradication rates of concomitant and sequential therapies were similar. However, the compliance with concomitant therapy was higher. Therefore, nonbismuth concomitant quadruple therapy should be the first-line treatment for *H. pylori* infection.

Lin LC, Hsu TH, Huang KW, Tam KW. Nonbismuth concomitant quadruple therapy for *Helicobacter pylori* eradication in Chinese regions: A meta-analysis of randomized controlled trials. *World J Gastroenterol* 2016; 22(23): 5445-5453 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i23/5445.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i23.5445

INTRODUCTION

Helicobacter pylori (*H. pylori*) infection has been proven to be the major cause of chronic gastritis, gastric and duodenal ulcers, gastric adenocarcinoma, and gastric mucosa-associated lymphoma[1-3]. Moreover, *H. pylori* eradication has become the standard and most widely adopted therapy for curing peptic ulcers[4-6].

According to most international guidelines, conventional triple therapy, involving the use of a proton pump inhibitor (PPI) with amoxicillin and clarithromycin for 7-10 d, is the first-line therapy for *H. pylori* eradication[7-10]. However, the eradication rate of triple therapy has decreased to 80% in many countries worldwide[11-14]. By contrast, studies have shown a high eradication rate for sequential therapy, which entails administering a PPI and amoxicillin for 5 d, followed by a PPI, clarithromycin, and metronidazole (or tinidazole) for another 5 d[15-18]. However, compliance may be poor because of the complexity of sequential therapy[19]. In addition, nonbismuth concomitant quadruple therapy, involving the simultaneous administration of a PPI, amoxicillin, clarithromycin, and metronidazole for 7 or 10 d, is more convenient than sequential therapy, although its efficacy is yet to be determined[20-26].

Peptic ulcer is a common disease in Chinese regions. In Taiwan, the overall prevalence of *H. pylori* infection is 54%, and it increases with age[27]. However, the infection rate of *H. pylori* is only 31% in Singapore[28]. Because antibiotic resistance is a critical reason for *H. pylori* eradication failure, studies on *H. pylori* eradication are needed within specific region[14]. However, most meta-analyses of *H. pylori* eradication have been performed in Europe and Korea, and the optimal treatment for *H. pylori* eradication in Chinese regions is still unknown[29,30]. Therefore, we conducted a systematic review and meta-analysis of randomized controlled trials (RCTs) to evaluate whether nonbismuth concomitant quadruple therapy is the first-line therapy for *H. pylori* eradication in Chinese regions.

MATERIALS AND METHODS

Data sources

The PubMed, Embase, Scopus, and Cochrane databases were searched for studies published in the period up to March 2016 without language restrictions. The following medical search heading terms, words, and combinations of words were used in the systematic search: *Helicobactor pylori* or *H. pylori, eradication, peptic* or *gastric* or *duodenal ulcer, concomitant* or *quadruple, and China* or *Chinese* or *Hong Kong* or *Taiwan* or *Singapore*. All included studies were also entered into the PubMed "similar articles" function and the science citation index. Moreover, we identified additional studies by manually searching the reference sections of these papers and by contacting known experts in the field. Finally, unpublished trials were retrieved from the ClinicalTrials.gov registry (http://clinicaltrials.gov/). The systematic review described herein was accepted by the online PROSPERO international prospective register of systematic reviews of the National Institute for Health Research (CRD42016-032668).

Study selection

The following studies were selected for analysis: RCTs evaluating the efficacy of nonbismuth concomitant quadruple therapy versus standard triple or sequential for *H. pylori* eradication; those performed in Chinese regions including China, Hong Kong, Taiwan, and Singapore; patients aged 18 years or over; those

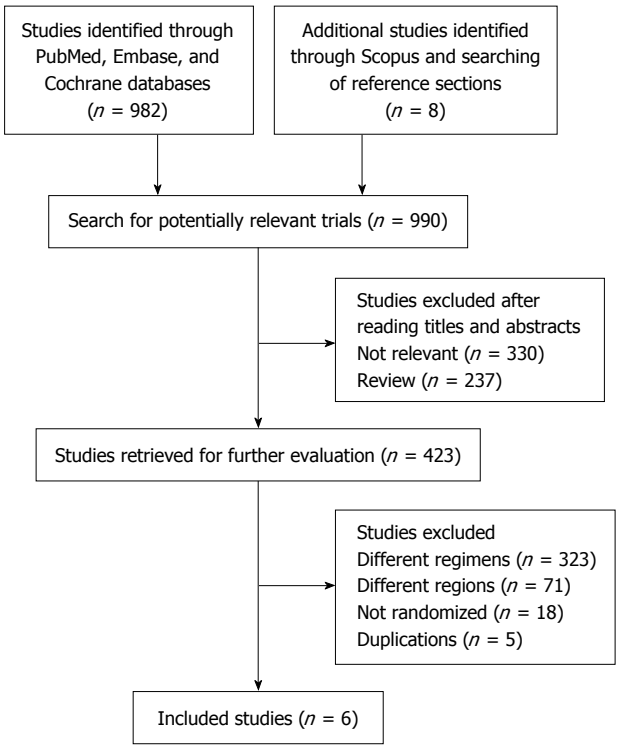

Figure 1 Flowchart for study selection.

clearly describing the inclusion and exclusion criteria used for patient selection; those adequately describing the administration of antibiotics and PPIs; and trials that precisely defined and evaluated *H. pylori* infection. Triple therapy was defined as a PPI plus amoxicillin and clarithromycin given for 7-14 d. Sequential therapy was defined as a PPI plus amoxicillin given for the first 5-7 d, followed by a PPI plus nitroimidazole derivatives and clarithromycin for the next 5-7 d. Nonbismuth concomitant quadruple therapy was defined as a PPI plus amoxicillin, clarithromycin, and nitroimidazole derivatives given for 7-14 d. The Studies were excluded from the analysis if one or both of the following criteria were present: patients enrolled in the trials who were proven to have had previous *H. pylori* infection with a history of bacterial eradication, and an overlap occurred between patient cohorts evaluated in two or more studies.

Data extraction and quality assessment

Two independent reviewers (Lin LC and Hsu TH) extracted the data of the trials, including the participants, inclusion and exclusion criteria, administration of experimental drugs, prevalence and assessment of *H. pylori* infection, and complications. Discrepancies and any disagreements were resolved through discussion with a third reviewer (Tam KW). The authors of the studies were contacted for additional information when necessary.

The risk of bias in the included trials was assessed using the following domains: adequacy of the randomization, allocation concealment, blinding, duration of follow-up, numbers of drop-outs, and performance of intention-to-treat (ITT) analysis.

Data synthesis and analysis

The *H. pylori* eradication rate was the primary outcome used to evaluate the efficacy of nonbismuth concomitant quadruple therapy. The occurrence of *H. pylori* infection was determined using assessments of histology, culture, rapid urease tests, or breath tests. The secondary outcome was the compliance with treatment.

The analysis was performed using the statistical package Review Manager, version 5.3 (Cochrane Collaboration, Oxford, England). The meta-analysis was performed according to the recommendations of the PRISMA statement[31]. For dichotomous data, the results were summarized as risk ratios (RRs) with 95%CIs. A pooled estimate of the RR was calculated using the DerSimonian and Laird random effect model[32]. This approach provides a more appropriate estimate of the average treatment effect when trials are statistically heterogeneous and usually yields wider CIs, thereby resulting in a more conservative statistical claim. χ^2 statistics tests (Q statistics) and the I^2 test were used to test for heterogeneity among controlled trials.

RESULTS

Characteristics of the trials

The review process is outlined in Figure 1. The initial search yielded 990 studies, 567 of which were deemed ineligible through screening of titles and abstracts. Subsequently, the full text of 423 studies was screened. Of these, five did not meet the eligibility criteria because of duplicate publication, 18 were not randomized studies, 71 evaluated *H. pylori* eradication in different regions, and 323 included different comparisons. Thus, only six eligible trials were included in this meta-analysis[20,22,33-36].

The main characteristics of the studies are listed in Table 1. Four of the six studies were performed in Taiwan, and the remaining two were conducted in China and Singapore. The publication dates of the studies were between 2012 and 2015, and the sample sizes ranged from 169 to 462. All trials evaluated patients diagnosed with *H. pylori* infection. In our included studies, three indicated that their patients had gastritis or peptic ulcers[33-35]. Only one study did not report the tests for diagnosing *H. pylori* infection[34], and other studies reported the following diagnostic tests: rapid urease test, histology, urea breath test, and culture. The timing of evaluation for the *H. pylori* infection status ranged from 4 to 12 wk after the treatment course. Treatment strategies for *H. pylori* eradication varied among the studies. Regarding the PPIs administered, three studies used esomeprazole[20,34,35], one used pantoprazole[33], one used lansoprazole[22], and one

Table 1 Characteristics of included studies

Ref.	Inclusion criteria	Region	Diagnostic test	No. of patients (male %)	Age, yr (mean ± SD)	Intervention
Ang *et al*[36] (2015)	Age > 21 yr	Singapore	RUT, H, UBT	C10: 153 (47.1)	C10: 46.9 ± 14.8	10-d concomitant therapy
				S10: 154 (59.7)	S10: 47.5 ± 12.7	10-d sequential therapy
				T10: 155 (58.1)	T10: 49.8 ± 14.6	10-d triple therapy
Hsu *et al*[33] (2014)	Age ≥ 20 yr, PU or gastritis	Taiwan	RUT, Cu, H	C7: 102 (59.8)	C7: 53.9 ± 12.3	7-d concomitant therapy
				S10: 102 (50.9)	S10: 55.0 ± 12.0	10-d sequential therapy
				T7: 103 (60.2)	T7: 56.1 ± 14.0	7-d triple therapy
Huang *et al*[22] (2012)	Dyspepsia or epigastric discomfort	Taiwan	RUT, Cu, H	C10: 84 (57.1)	C10: 53.8 ± 15.2	10-d concomitant therapy
				S10: 85 (56.7)	S10: 51.3 ± 15.0	10-d sequential therapy
Tai *et al*[34] (2015)	Age ≥ 20 yr, PU or gastritis	Taiwan	Not reported	C7: 92 (50.0)	C7: 47.8 ± 11.6	7-d concomitant therapy
				T7: 92 (49.0)	T7: 52.8±12.8	7-d triple therapy
Wang *et al*[35] (2014)	PU and gastritis	China	UBT	C7: 81 (45.7)	C7: 51 ± 13	7-d concomitant therapy
				T7: 82 (42.7)	T7: 51 ± 15	7-d triple therapy
				T10: 83 (45.8)	T10: 52 ± 14	10-d triple therapy
Wu *et al*[20] (2010)	Patients visited GI clinics with HP infection	Taiwan	RUT, Cu, H	C10: 115(52.2)	C10: 51.8 ± 11	10-d concomitant therapy
				S10:117(52.1)	S10: 51.7 ± 12	10-d sequential therapy

Concomitant therapy: PPI, amoxicillin, clarithromycin, and metronidazole for 7-10 d; sequential therapy: PPI and amoxicillin for 5 d, followed by PPT, clarithromycin, and metronidazole (or tinidazole) for 5 d; triple therapy: PPI, amoxicillin, and clarithromycin for 7-10 d. PPI: lansoprazole, pantoprazole, or esomeprazole; GI: Gastrointestinal; PU: Peptic ulcer; C7: 7-d concomitant therapy; C10: 10-d concomitant therapy; S10: 10-d sequential therapy; T7: 7-d triple therapy; T10: 10-d triple therapy; Cu: Culture; H: Histology; RUT: Rapid urease test; UBT: Urea breath test.

did not control the choice of the PPI[36]. Regarding concomitant and sequential regimens, all studies used metronidazole, except for Wang *et al*[35], who substituted metronidazole with tinidazole. In all included studies, a PPI, amoxicillin, and clarithromycin were administered as triple therapy for 7 d[33-35] or 10 d[35,36]. Regarding sequential therapy, all treatment regimens entailed administering a PPI and amoxicillin for 5 d, followed by a PPI, clarithromycin, and metronidazole for another 5 d[20,22,33,36]. Finally, concomitant therapy involved administering a PPI, clarithromycin, amoxicillin, and metronidazole for 7 d[33-35] or 10 d[20,22,36]. Huang *et al*[22] prolonged PPI maintenance therapy to 10 wk. Baseline characteristics were balanced and similar between groups in the six included RCTs.

Table 2 presents the details of the six included RCTs. The use of random allocation was clearly documented in all studies. The treatment group allocation was concealed from the patients in three studies[33,34,36]. Only one reported the blinding of the investigators who assessed the outcomes[20]. In all studies, outcomes were evaluated using both ITT and per-protocol analyses. The percentage of patients lost to follow-up was acceptable (< 20%) in all studies. All studies had a bias attributable to insufficient data on antibiotic susceptibility[20,22,33,36].

H. pylori eradication rate

Nonbismuth concomitant quadruple therapy *vs* triple therapy: Three studies compared the *H. pylori* eradication rates of 7-d nonbismuth concomitant quadruple and triple therapies[33-35]. The timing of *H. pylori* infection status assessment was different among these studies: 4[35], 6[33], and 8 wk[34] after treatment. A significant difference was observed in the overall *H. pylori* eradication rate of nonbismuth concomitant quadruple and triple therapies (91.2% *vs* 77.9%). Fewer patients receiving nonbismuth concomitant quadruple therapy experienced *H. pylori* infection after treatment (RR = 1.17, 95%CI: 1.09-1.25) (Figure 2). The results demonstrated low heterogeneity among the studies (I^2 = 0%).

One study compared the *H. pylori* eradication rates of 10-d nonbismuth concomitant quadruple and triple therapies[36]. The timing of *H. pylori* infection status assessment was 4 wk after treatment. No significant difference was observed in the *H. pylori* eradication rates of nonbismuth concomitant quadruple and triple therapies (81.7% *vs* 83.2%, RR = 0.98, 95%CI: 0.89-1.09) (Figure 2).

Nonbismuth concomitant quadruple therapy *vs* sequential therapy: Three studies compared the *H. pylori* eradication rate of 10-d nonbismuth concomitant quadruple and sequential therapies[20,22,36]. The timing of *H. pylori* infection status assessment was different among the studies: 4[36], 6[20], and 12 wk[22] after treatment. No statistically significant difference was observed in the overall *H. pylori* eradication rates of nonbismuth concomitant quadruple and sequential therapies (86.9% *vs* 86.0%, RR = 1.01, 95%CI: 0.95-1.07) (Figure 2).

Compliance

Nonbismuth concomitant quadruple therapy *vs* triple therapy: Three studies compared the compliance with 7-d nonbismuth concomitant quadruple and triple therapies[33-35]. No statistically significant difference was observed in the compliance with these therapies (100% *vs* 99.3%, RR = 1.01, 95%CI: 0.99-1.02) (Figure 3).

Nonbismuth concomitant quadruple therapy *vs* sequential therapy: Three studies compared

Table 2 Assessment of methodological quality of included studies

Ref.	Region	Allocation generation	Allocation concealment	Blinding of patients and assessors	Data analysis	Loss to follow up	Selective reporting	Other bias
Ang *et al*[36] (2015)	Singapore	Sealed envelope	Adequate	Open-label	ITT/PP	10.0%	Low risk	Not all patients underwent antibiotic susceptibility testing
Hsu *et al*[33] (2014)	Taiwan	Compute generated	Adequate	Open-label	ITT/PP	0.3%	Low risk	Not all patients underwent antibiotic susceptibility testing
Huang *et al*[22] (2012)	Taiwan	Computer generated	Unclear	Open-label	ITT/PP	6.5%	Low risk	No patient underwent antibiotic susceptibility testing
Tai *et al*[34] (2015)	Taiwan	Computer generated	Adequate	Unclear	ITT/PP	8.0%	Low risk	Not all patients underwent antibiotic susceptibility testing
Wang *et al*[35] (2014)	China	Computer generated	Unclear	Unclear	ITT/PP	1.2%	Low risk	No patient underwent antibiotic susceptibility testing
Wu *et al*[20] (2010)	Taiwan	Computer generated	Unclear	Outcome assessor blinded	ITT/PP	0.4%	Low risk	Not all patients underwent antibiotic susceptibility testing

Risk of bias was assessed according to the method recommended by the Cochrane Collaboration. ITT: Intention-to-treat; PP: Per-protocol.

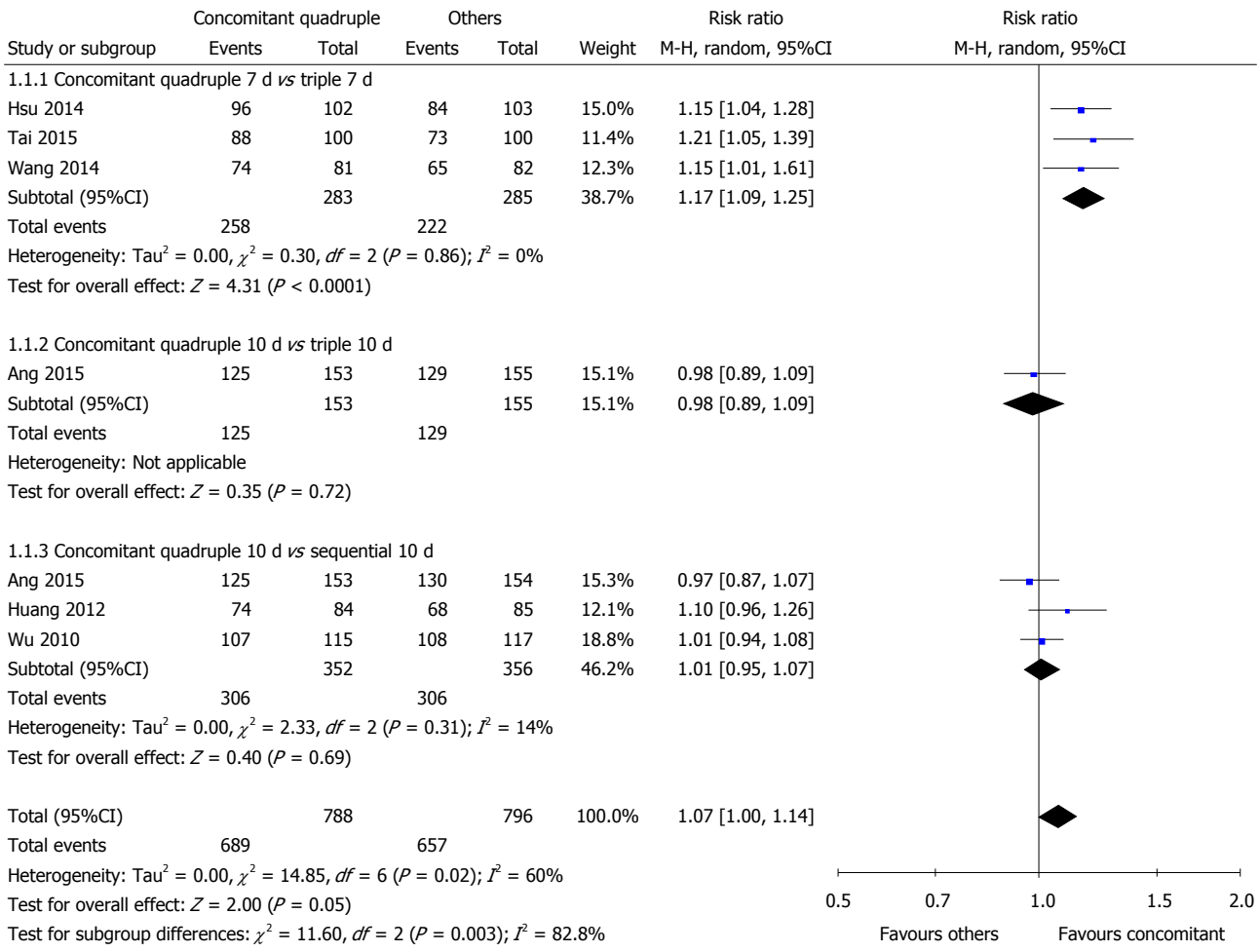

Figure 2 Forest plot for comparison of concomitant quadruple therapy with other therapies. Outcome: *Helicobacter pylori* eradication rate.

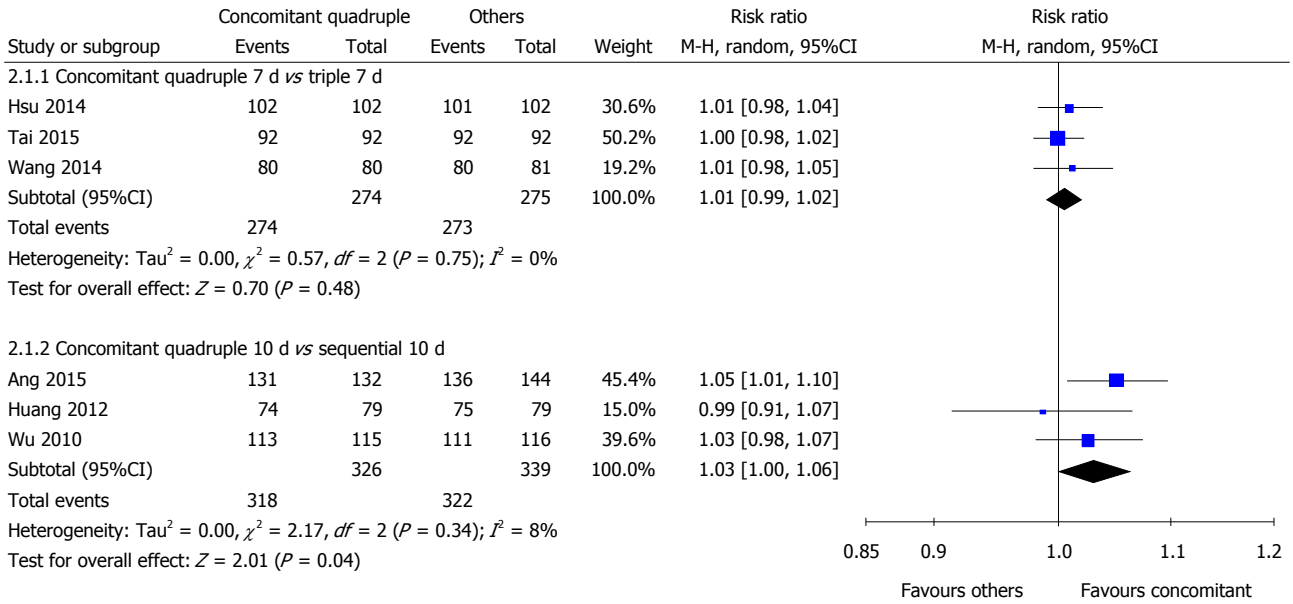

Figure 3 Forest plot for comparison of concomitant quadruple therapy with other therapies. Outcome: Compliance.

the compliance with 10-d nonbismuth concomitant quadruple and sequential therapies[20,22,36]. Although no significant difference was observed in the compliance with these therapies (97.5% *vs* 95.0%, RR = 1.03, 95%CI: 1.00-1.06), more patients tended to comply with nonbismuth concomitant quadruple therapy (Figure 3).

Adverse events

Two studies compared adverse events including abdominal pain, gastrointestinal disturbance, nausea and vomiting, skin rash, dizziness, and fatigue between 10-d nonbismuth concomitant quadruple and sequential therapies[20,22]. Patients receiving these two therapies showed a similar adverse event rate. Moreover, three studies compared the adverse event rate between 7-d nonbismuth concomitant quadruple and triple therapies[33-35]. Among two studies, patients receiving these two therapies showed a similar incidence of adverse events[33,35]. One study reported more adverse events after 7-d nonbismuth concomitant quadruple therapy than after triple therapy[34]. However, these effects were mild and did not markedly interfere with the patients' daily activities.

DISCUSSION

Because antibiotic resistance is a critical reason for *H. pylori* eradication failure, we conducted a systematic review and meta-analysis of RCTs to evaluate whether nonbismuth concomitant quadruple therapy is the optimal first-line therapy for *H. pylori* eradication in Chinese regions. Our meta-analysis revealed that a higher *H. pylori* eradication rate was achieved with 7-d concomitant therapy than with 7-d triple therapy. The eradication rates of concomitant and sequential therapies were similar. However, the compliance with concomitant therapy was higher. Therefore, nonbismuth concomitant quadruple therapy should be the first-line treatment for *H. pylori* infection.

Recently, Li *et al*[37] conducted a network meta-analysis of treatment for *H. pylori* infection. They showed that nonbismuth concomitant quadruple treatment is effective in *H. pylori* eradication. However, ethnicity and region play pivotal roles in antibacterial treatments; thus, investigating *H. pylori* eradication in different regions is necessary[38]. In South Korea, two RCTs showed that a much higher *H. pylori* eradication rate was achieved with nonbismuth concomitant quadruple therapy than with standard triple therapy or sequential therapy[26,39]. In Japan, an RCT also reported a higher eradication rate for nonbismuth concomitant quadruple therapy than that for triple therapy[40]. Our study revealed a similar outcome in Chinese regions.

Although the included studies used different PPIs, the same PPI was administered to the experimental groups in all studies, except for Ang *et al*[36]. Nevertheless, a meta-analysis revealed that different PPI types did not have different efficacies for *H. pylori* eradication[41]. Moreover, regarding concomitant therapy, Wang *et al*[35] substituted metronidazole with tinidazole; the eradication rate of that study is similar to that of other studies using metronidazole. These results are compatible with the trial that compared the efficacy of tinidazole and metronidazole for *H. pylori* eradication[42].

The optimal dosage of metronidazole remains undetermined. All RCTs used 500 mg of metronidazole twice daily, except for Ang *et al*[36], who used 400 mg twice daily. Nevertheless, Ang *et al*[36] still obtained a high eradication rate; this finding indicated that metronidazole doses from 400 to 500 mg are acceptable for *H.*

pylori eradication.

To determine the effectiveness of the treatments in practice, we considered the compliance rate. Although the eradication rates of nonbismuth concomitant and sequential therapies were not statistically different, higher compliance was achieved with nonbismuth concomitant therapy than with sequential therapy. Generally, the compliance rate may be higher in RCTs than in clinical settings. Thus, nonbismuth concomitant therapy may be a superior choice for *H. pylori* eradication because higher compliance was achieved with this therapy than with sequential therapy.

The value of I^2 was 0%-14% for each therapy, revealed that mild heterogeneity existed among our selected studies. This could be attributed to heterogeneity among patients' demographics and characteristics and the inclusion and exclusion criteria, dose and route of administration of *H. pylori* treatment, and time of outcome assessment.

Our study has several limitations. First, all our studies were open label, except for the study of Wu *et al*[20], which was outcome assessor blinded. However, we believe that this is not a major concern because the treatment outcomes were mainly objective. Second, not all trials evaluate antibiotic susceptibility. Third, because China has the highest population globally, more RCTs conducted in China may be required to determine the optimal treatment for *H. pylori* infection in Chinese regions.

In conclusion, the evidence reviewed in the present meta-analysis indicated that nonbismuth concomitant quadruple therapy achieved a higher *H. pylori* eradication rate than that of standard triple therapy and higher compliance than that of sequential therapy. Therefore, nonbismuth concomitant quadruple therapy should be the first-line treatment for *H. pylori* infection in Chinese regions.

COMMENTS

Background

Peptic ulcer is a common disease in Chinese regions, and *Helicobacter pylori (H. pylori)* eradication has become the standard and most widely adopted therapy. However, eradication rate of standard triple therapy has decreased to 80% in many countries worldwide, and compliance of sequential therapy may be poor due to the complexity. Therefore, there is a need to evaluate whether nonbismuth concomitant quadruple therapy is the first-line therapy for *H. pylori* eradication in Chinese regions.

Research frontiers

Due to increasing antibiotic resistance, current *H. pylori* eradication therapies may be poor. In this study, the authors compared eradication rate and compliance rate of three different types of therapies in Chinese region.

Innovations and breakthroughs

This study is the first meta-analysis to compare nonbismuth concomitant quadruple therapy with triple therapy and sequential therapy in Chinese region. Based on this study, nonbismuth concomitant quadruple therapy showed high *H. pylori* eradication rate and good compliance rate in Chinese region.

Applications

This study showed high efficacy and compliance in nonbismuth concomitant quadruple therapy. Thus, nonbismuth concomitant quadruple therapy is a good choice for first-line *H. pylori* eradication therapy in Chinese region.

Terminology

Nonbismuth concomitant quadruple therapy consist of proton pump inhibitor plus amoxicillin, clarithromycin, and nitroimidazole derivatives given for 7-14 d. Chinese regions in this study included China, Hong Kong, Taiwan, and Singapore.

Peer-review

The results of this meta-analysis showed that treatment with nonbismuth concomitant quadruple therapy resulted in high *H. pylori* eradication rate in Chinese region. In addition, good compliance rate and mild adverse effects were also noted in this study. Consequently, the study provided a better choice for first-line eradication therapy of *H. pylori* in Chinese region.

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CASE REPORT

Endoscopic *en bloc* resection of an exophytic gastrointestinal stromal tumor with suction excavation technique

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Author contributions: Chun HJ designed the report; Kim ES, Keum B, Jeen YT, Lee HS and Kim CD collected the patient's clinical data; Choi HS and Kim KO analyzed the data and wrote the paper.

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Conflict-of-interest statement: The authors have no conflicts of interest to declare.

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Abstract

Here, we report the first successful endoscopic resection of an exophytic gastrointestinal stromal tumor (GIST) using a novel perforation-free suction excavation technique. A 49-year-old woman presented for further management of a gastric subepithelial tumor on the lesser curvature of the lower body, originally detected *via* routine upper gastrointestinal endoscopy. Abdominal computed tomography and endoscopic ultrasound showed a 4-cm extraluminally protruding mass originating from the muscularis propria layer. The patient firmly refused surgical resection owing to potential cardiac problems, and informed consent was obtained for endoscopic removal. Careful dissection and suction of the tumor was repeated until successful extraction was achieved without serosal injury. We named this procedure the suction excavation technique. The tumor's dimensions were 3.5 cm × 2.8 cm × 2.5 cm. The tumor was positive for C-KIT and CD34 by immunohistochemical staining. The mitotic count was 6/50 high-power fields. The patient was followed

for 5 years without tumor recurrence. This case demonstrated the use of endoscopic resection of an exophytic GIST using the suction excavation technique as a potential therapy without surgical resection.

Key words: Gastrointestinal stromal tumor; Endoscopic resection; Submucosal tumor; Subepithelial tumor; *En bloc* resection

Core tip: Most small gastrointestinal stromal tumor (GISTs) are treated surgically, and endoscopic resection is contraindicated as the risk of perforation and incomplete resection is high. However, several authors recently reported successful results with endoscopic resection of a subepithelial tumor originating from the muscularis propria layer, including GISTs. A GIST with exophytic growth was previously considered a contraindication for endoscopic resection. In cases of exophytic GISTs, surgical or endoscopic full-thickness resection with laparoscopic support is generally indicated. However, this case shows that even exophytic GISTs can potentially be endoscopically resected without perforation using the suction excavation method.

Choi HS, Chun HJ, Kim KO, Kim ES, Keum B, Jeen YT, Lee HS, Kim CD. Endoscopic *en bloc* resection of an exophytic gastrointestinal stromal tumor with suction excavation technique. *World J Gastroenterol* 2016; 22(23): 5454-5458 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i23/5454.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i23.5454

INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumor of the gastrointestinal tract, especially of the stomach. Most small GISTs are treated surgically, and endoscopic resection was previously contraindicated as the risk of perforation and incomplete resection is high. Recently, however, several authors have reported successful results using endoscopic resection for subepithelial tumors (SETs) originating from the muscularis propria layer, including GISTs[1-3]. However, GISTs with exophytic growth have been considered a contraindication for endoscopic resection. Few studies on endoscopic resection of an extragastric bulging SET have been published, and the procedure reported previously was full-thickness resection of the gastric wall with laparoscopic support. In cases of exophytic GISTs, surgical or endoscopic full-thickness resection with laparoscopic support is usually indicated. However, the case presented here shows that even exophytic GISTs can potentially be endoscopically resected without perforation. We report the first successful endoscopic resection of a GIST with exophytic growth using a suction excavation technique.

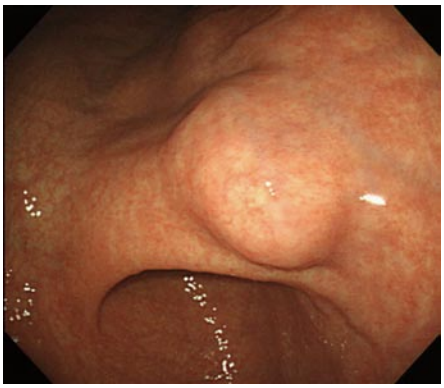

Figure 1 Endoscopic view of the lesion.

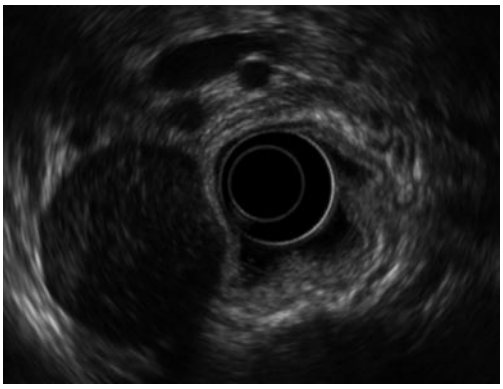

Figure 2 Endoscopic ultrasound. Endoscopic ultrasonography showed a heterogenic hypoechoic mass with extraluminal growth originating from the muscularis propria layer.

CASE REPORT

A 49-year-old woman visited our hospital for further evaluation and management of a gastric SET, detected *via* routine upper gastrointestinal endoscopy at a private health clinic. The esophagogastroduodenoscopy performed outside the hospital showed a 4-cm mass with a smooth tapered margin located in the lower body, on the side of lesser curvature (Figure 1). The mass was hard and immovable upon palpation with biopsy forceps. Endoscopic ultrasound showed a heterogeneous hypoechoic mass with extraluminal growth, originating from the muscularis propria layer (Figure 2). An abdominal CT scan showed a 4-cm extraluminally protruding mass compressing the gastric wall (Figure 3). Our initial treatment plan was surgical resection owing to the tumor's extraluminal growth pattern. However, the patient resolutely refused surgical resection and we therefore chose to attempt an endoscopic removal of the tumor. The endoscopic submucosal dissection procedure was performed using a cap-fitted gastroscope (GIF-Q260; Olympus, Tokyo, Japan). The endoscopic procedure is clearly illustrated and presented in Figure 4. Marking was done along

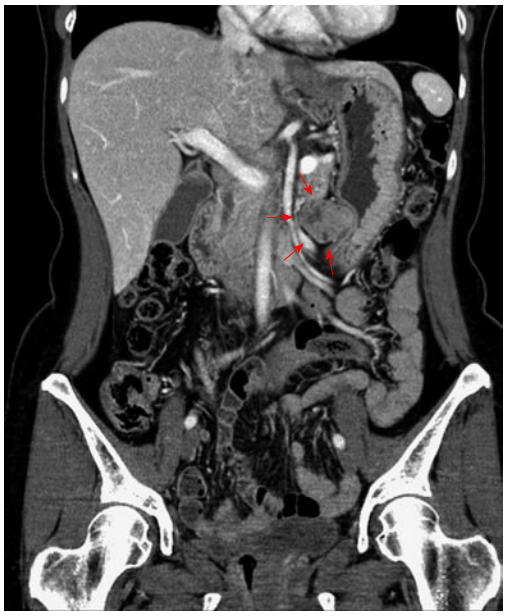

Figure 3 Abdominal computerized tomography scan showing the 3.5 cm × 2.5 cm tumor with exophytic growth.

the tumor's margin using an argon plasma coagulation probe. After submucosal injection of sodium hyaluronate, a circumferential incision was made along the marking line using a hook knife (KD-620Q; Olympus). After the circumferential incision, the overlying mucosa was removed by snare resection and dissection of the muscular tissue between the tumor and the serosa (Figure 5). Muscular dissection was carefully performed using a hook knife. Shortly after muscular dissection, suction was applied to the tumor using the end of the cap-fitted gastroscope. This careful dissection and suction of the tumor was repeated until, finally, the tumor was successfully extracted without serosal injury (Figure 6). The procedure time was approximately 1 h. Propofol was initially injected intravenously to induce sedation (0.5 mg/kg), and additional propofol was administered repeatedly during the endoscopic procedure (10-20 mg per each injection). Supplemental oxygen was administered nasally throughout sedation.

The tumor dimensions were 3.5 cm × 2.8 cm × 2.5 cm (Figure 7). Immunohistochemical staining showed that the tumor was positive for C-KIT and CD34. It was diagnosed as GIST. The mitotic count was 6/50 high-power fields. Although the patient had a high risk of recurrence according to risk stratification analysis, further surgery could not be performed owing to the patient's unwillingness to undergo surgical resection. No tumor recurrence occurred during the 5-year follow-up period (Figure 8).

DISCUSSION

The number of clinical trials investigating endoscopic resection of SETs originating from the muscularis propria layer has recently increased. These trials have reported that some SETs originating from the muscularis propria layer can be safely resected by using gastrointestinal endoscopy without perforation or any other serious complications. However, it is not yet known which of these tumors is an appropriate indication for endoscopic resection. Appropriate indications for endoscopic resection should be based on low complication and recurrence rates. A previous study showed that a positive rolling sign and a small tumor size are appropriate indications for endoscopic removal. In addition, successful endoscopic resection of GISTs depends on their location in the gastric wall[4]. Submucosal tumors that protrude mainly into the serosal side of the gastric wall are not easy to resect completely using an endoscopic method. Nevertheless, further studies are required to verify these findings. In cases of exophytic GISTs, surgical or endoscopic full-thickness resection with laparoscopic support is generally indicated. However, this case shows that even exophytic GISTs can potentially be endoscopically resected without perforation.

Previous endoscopic therapy did not emphasize the suction concept, but we showed that suction excavation is useful for SET removal. Full endoscopic suction can move SETs in an endoluminal direction, so our technique has the advantage of reducing perforation risk compared with previous endoscopic submucosal dissection techniques. However, the suction excavation technique has its own limitations. If the SETs do not move inward by endoscopic suction, this technique cannot be used for endoscopic resection. Therefore, it is difficult to apply our suction excavation technique to all exophytic SET resections. The use of the suction excavation technique to remove exophytic SETs has limited indications, such as a positive rolling sign, small tumor size, and moving sign by endoscopic suction. However, the suction excavation technique can be useful for removing simple SETs as well as exophytic SETs in clinical practice.

Most cases of recurrence after surgical resection of a GIST involve intraperitoneal or hepatic recurrence. Local recurrence itself is very rare. The risk factors of GIST recurrence include not only tumor size and the number of mitoses but also exposure of tumor cells in the abdominal cavity after the capsule of the tumor is ruptured[5]. If gastric perforation can be avoided during endoscopic resection, the risk of tumor cell exposure in the abdominal cavity could be lower than that of surgical resection and the frequency of intra-abdominal recurrence would decrease accordingly. As such, avoidance of gastric perforation during endoscopic resection is critical. In this case, the patient was followed for 5 years without tumor recurrence. A recent study comparing the results of endoscopic versus surgical resection of a GIST in the upper gastrointestinal tract found that endoscopic resection could be an alternative therapeutic modality in selective cases[5]. In our subject's case, the tumor showed

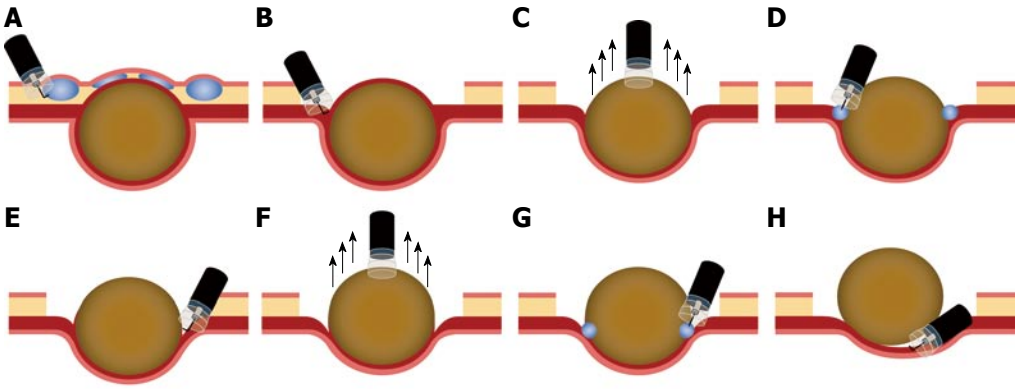

Figure 4 Illustration of the endoscopic suction excavation technique. A: After submucosal injection of sodium hyaluronate, a circumferential incision and removal of the overlying mucosa were performed; B, E: Careful muscular dissection between the tumor and serosa with a hook knife; C, F: Endoscopic suction using the end of a cap-fitted endoscope attached to the tumor; D, G: Submucosal injection of sodium hyaluronate; H: Tumor fully extracted after repeated careful dissection and endoscopic suction.

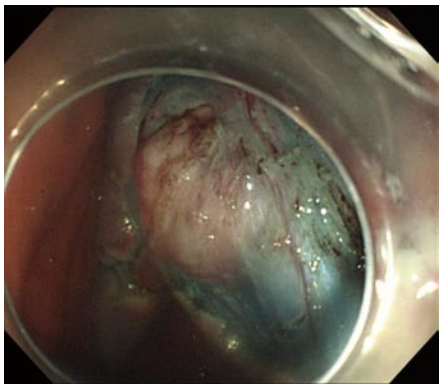

Figure 5 Tumor was exposed after removing the overlying mucosa.

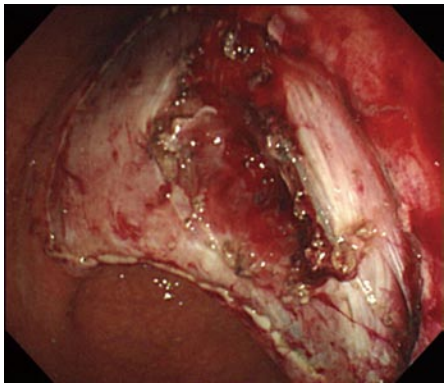

Figure 6 Extraction showing no perforation.

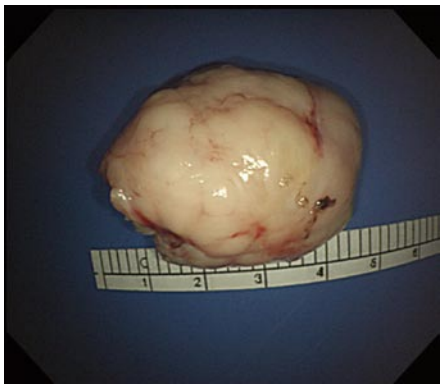

Figure 7 Extracted tumor after repeated muscular dissection and endoscopic suction.

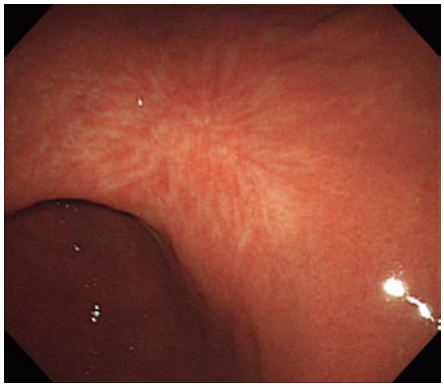

Figure 8 Endoscopic submucosal dissection scar after 5 years.

extraluminal bulging or exophytic growth, and thus, the risk of perforation during endoscopic resection was very high. This suction excavation method can be safer than endoscopic submucosal dissection. Although this method does require physician experience, it can be helpful in difficult cases of endoscopic GIST removal.

In the case presented here, a full-thickness resection with laparoscopic support followed by laparoscopic gastroparietal suture, as opposed to an endoscopic-only procedure, is usually indicated. In this case, endoscopic resection of the extraluminal bulging SET using a suction excavation technique without laparoscopic support was successfully performed for the first time. Although not all exophytic GISTs can be resected endoscopically using this excavation technique, we do hope that this method may aid in the removal of tough submucosal tumors using gastrointestinal endoscopy.

COMMENTS

Case characteristics

A 49-year-old woman presented for further management of a gastric subepithelial tumor on the lesser curvature of the lower body, originally detected *via* routine upper gastrointestinal endoscopy.

Clinical diagnosis

The esophagogastroduodenoscopy performed outside the hospital showed a 4-cm mass with a smooth tapered margin located in the lower body, on the side of lesser curvature. The mass was hard and immovable upon palpation with biopsy forceps.

Differential diagnosis

Subepithelial tumor: Gastrointestinal stromal tumor, leiomyoma, granular cell tumor, ectopic pancreas.

Laboratory diagnosis

All labs were within normal limits.

Imaging diagnosis

Endoscopic ultrasound showed a heterogeneous hypoechoic mass with extraluminal growth, originating from the muscularis propria layer. An abdominal CT scan showed a 4-cm extraluminally protruding mass compressing the gastric wall.

Pathological diagnosis

Immunohistochemical staining showed that the tumor was positive for C-KIT and CD34. It was diagnosed as gastrointestinal stromal tumors (GISTs). The mitotic count was 6/50 high-power fields.

Treatment

Endoscopic resection of an exophytic GIST using the suction excavation technique.

Related reports

Most GISTs are treated surgically, and endoscopic resection was contraindicated as the risk of perforation and incomplete resection is high. However, several authors have recently reported successful results using endoscopic resection of a subepithelial tumor (SET) originating from the muscularis propria layer, including GISTs. A GIST with exophytic growth was previously considered a contraindication for endoscopic resection. In cases of exophytic GISTs, surgical or endoscopic full-thickness resection with laparoscopic support is generally indicated. However, this case shows that even exophytic GISTs can potentially be endoscopically resected without perforation using the suction excavation method.

Term explanation

GISTs are the most common mesenchymal neoplasms of the gastrointestinal tract that arise in smooth muscle pacemaker cells such as interstitial cells of Cajal.

Experiences and lessons

The suction excavation technique described here is a helpful technique to remove exophytic SETs. Previous endoscopic therapy did not emphasize the suction concept, but we showed that suction excavation is useful for SET removal.

Peer-review

The paper is well written.

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TOPIC HIGHLIGHT

2016 Hepatitis B virus: Global view

Natural regression of fibrosis in chronic hepatitis B

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Abstract

The fibrosis of liver cirrhosis was considered to be irreversible before the anti-viral drugs showed that it is reversible when they lead to continuous suppression of viral replication and inflammation. However, several reports previously showed that fibrosis of type B liver cirrhosis was almost completely absorbed after the natural remission of chronic inflammation. This phenomenon might not be limited to exceptional patients, but rather occur commonly, considering the dynamic clinical features of chronic hepatitis B (CHB), where inactive carrier stage normally follows aggravation of hepatitis and progression of fibrosis at the time of HBeAg seroconversion. Thus, fibrosis levels of CHB as a hepatocellular carcinoma (HCC)-surveillance marker, particularly those of the inactive stage, could be underestimated, because some of them might have been (pre)cirrhotic in the past and recovered with the natural regression of fibrosis. We argue that cirrhosis-induced HCC mechanisms, rather than direct action of viral genome, may be more common than generally considered in CHB patients. This may have some impact on reconsidering the surveillance rationale for HCC in CHB, from where advanced HCCs tended to be missed. In addition, a molecular marker to assess the cancer-prone characteristics of the liver will definitely be needed to resolve the issue.

Key words: Chronic hepatitis B; Cirrhosis; Spontaneous remission; Regression of fibrosis; Occult hepatitis B infection; Hepatocellular carcinoma surveillance of hepatitis B virus

Core tip: The fibrosis of liver cirrhosis may be reversible. Regression of fibrosis in hepatitis B virus (HBV) patients with (pre)cirrhosis might be a more common phenomenon than generally considered. This might cause the underestimation of fibrosis levels in chronic hepatitis B, suggesting a difficulty with the surveillance system of HBV-hepatocellular carcinoma (HCC). That is, some HCC patients with non-cirrhotic liver might have been cirrhotic in the past, after which spontaneous regression of fibrosis occurred. Cirrhosis-HCC mechanisms, compared to the direct action of the viral genome, might be more prevalent than generally considered in HBV patients.

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INTRODUCTION

Continuous inflammation of the liver induces deposition of extracellular matrix (ECM) and results in liver cirrhosis. Although several reports showed regression of fibrosis in animal models with liver cirrhosis[1,2], and in human case reports[3-5], established fibrosis of cirrhosis was generally considered to be irreversible[6,7]. However, reversibility of fibrosis in the liver became commonly-known recently because elimination of viruses with anti-hepatitis B virus (HBV) or hepatitis C virus (HCV) drugs caused regression of fibrosis even in cirrhotic cases[8,9].

The natural history of chronic HBV infection pursues a dynamic process caused by the conflict between virus and immune system, which results in the seroconversion of HBeAg to anti-HBe, followed by that of HBsAg to anti-HBs[10]. Long-standing liver injury in the process of HBeAg-seroconversion induces particularly rapid progression of fibrosis that frequently results in cirrhosis. However, due to the dynamic course of the disease, stable clinical remission with low HBV DNA and transaminase levels follows, and the majority of patients become inactive carriers. Natural regression of fibrosis induced by this clinical remission has been reported[3,11].

In general, hepatocellular carcinoma (HCC) with HBV arises more frequently in non-cirrhotic liver than in HCV infection[12,13]. This is considered mainly due to the direct mutagenesis effect of the HBV genome that integrates into the host genome or the direct hepatocarcinogenic action of viral genes such as hepatitis B virus X (HBx)[14]. However, if HCCs have arisen in the liver in which fibrosis naturally regressed from advanced liver fibrosis, they might be misinterpreted as having arisen from a non-cirrhotic liver.

Consequently, it should always be kept in mind that fibrosis levels of chronic hepatitis B (CHB) always have the possibility of being underestimated in terms of HCC-risk, because they might have regressed from a more advanced stage of fibrosis[15]. We attempt to spotlight the natural regression of fibrosis in chronic HBV infection and re-evaluate the clinical background of HBV-induced HCC in this review.

CHRONIC HBV DISEASE AND PROGRESSION OF FIBROSIS

About 350 million people in the world have chronic HBV infection[16]. Chronic HBV infection is a significant cause of liver cirrhosis, which bears a high risk of HCC. The annual rate of development of HCC has been reported to be 10% to 17% in HBV-induced liver cirrhosis[17]. In general, fibrosis levels correlate well with the risk of HCC, as in the case with HCV infection.

The immunological attack against hepatocytes infected with HBV induces a dynamic clinical course which is represented by seroconversion of HBeAg to anti-HBe. Almost no or very mild liver injury is observed in the HBeAg-positive immunotolerant phase[18]. The severity of liver injury and the progression of fibrosis depend on how long and severe the immunological attacks continue in the phase of HBeAg seroconversion. A rapid transition to anti-HBe normally results in a silent clinical course, referred to as the inactive carrier state, while a difficult transition that is accompanied by aggravation of chronic hepatitis result in liver cirrhosis or HBeAb-positive chronic active hepatitis, which frequently bears core-promoter mutations. Importantly, the majority of patients become inactive carriers afterwards, irrespective of their clinical course[10,19].

MECHANISM OF HBV-INDUCED HCC AND CLINICAL BACKGROUND CHARACTERISTICS

Chronic inflammation induces a progression of fibrosis resulting in liver cirrhosis in which HCC frequently develops. This cirrhosis-HCC mechanism is common to both HBV and HCV infections and is considered to play the main role in hepatocarcinogenesis, to which a process of accumulation of genetic mutations and epigenetic events contributes. In addition to this common carcinogenetic mechanism, each virus infection has its own carcinogenic mechanism.

On the other hand, HBV, which is related to retroviruses, integrates into the host genome during the process of replication and acts as a mutagen. In addition, the *HBx* gene, which is a pleiotropic transactivator of many genes, has been known to be directly involved in hepatocarcinogenesis[20]. Because "cirrhosis to HCC" (indirect) and these

Table 1 A comparison of clinic-pathological data between type-B and type-C hepatocellular carcinoma

	Type B (*n* = 97)	Type C (*n* = 81)	*P* value
Age (yr)	56.1 ± 11.9	66.7 ± 9.8	< 0.0001
Sex (M:F)	71.1%:28.9%	60.5%:39.5%	0.18
AST (IU/L)	82.2 ± 98.6	81.8 ± 44.1	0.18
ALT (IU/L)	67.7 ± 70.9	83.2 ± 56.9	0.11
T.Bil (mg/dL)	1.2 ± 1.1	1.0 ± 0.6	0.36
Alb (g/dL)	4.0 ± 0.6	3.9 ± 0.5	0.22
Plt (/mm^3)	13.9 ± 8.1	10.8 ± 5.5	< 0.005
Histology			
F1:2:3:4	2:20:3:32	1:6:8:21	< 0.005
A1:2:3	17:25:12	1:14:17	< 0.005
Clinical stage (Ⅰ:Ⅱ:Ⅲ:Ⅳ)	12:29:25:31	15:32:29:5	< 0.0005

direct mechanisms are not mutually exclusive, it is unknown how much a role each mechanism plays in real hepatocarcinogenesis, especially in individual HCC patients with variable clinical backgrounds.

DIFFICULTY OF HCC SURVEILLANCE IN CHRONIC HBV PATIENTS

Because the carcinogenic processes of HBV and HCV are different, the rationale to perform effective cancer surveillance should be confirmed based on the difference in the specific clinical courses of these infections. Age, sex, fibrosis levels, the presence of HBeAg, HBV viral load, HBsAg levels accounts for the risks of HBV-HCC[21]. In reports from Japan, HCC patients with HBV tended to be younger and have advanced tumor when diagnosed, as well as lower fibrosis levels with good liver function when compared to those with HCV[22,23]. Our result also showed that patients with HBV were younger, with lower fibrosis levels and higher platelet counts than those with HCV (Table 1). Notably, the clinical stages of HBV-HCC were more advanced. These clinical features suggest that cancer surveillance of HBV is much more difficult than that of HCV infection, where continuous elevation of transaminase values induces gradually increasing fibrosis levels, and a low platelet count may be associated with advanced fibrosis levels[24]. In general, a direct hepatocarcinogenic mechanism of HBV, such as the integration of the genome or a direct role of an HBV gene such as HBx, may be considered to be involved in the occurrence of HCC in these "unexpected" patients. However, our question is "Is this the full explanation?"

It seems very paradoxical that HBeAg-positive carriers in the immunotolerant phase with high viral loads and little inflammation, who are supposed have abundant viral gene expression and integration event, seldom suffer from HCC[18]. On the other hand, most HCC patients who were younger than 20 years old were already seroconverted to anti-HBe, and the majority of them were cirrhotic[25-27]. Thus, the cirrhosis to HCC mechanism might play a central role even in these young HCC patients. These facts render the HCC surveillance system for HBV more complex.

REVERSIBILITY OF FIBROSIS WITH LIVER CIRRHOSIS WITH ANTI-HBV TREATMENT AND NATURAL COURSE

The generally accepted idea was that the fibrosis of liver cirrhosis is an irreversible process, and established fibrosis could not regress. However, the development of innovative anti-viral drugs for HBV and HCV changed this long-lasting concept[8]. Fibrosis is reversed when inflammation is suppressed by effective anti-viral agents, and even cirrhosis can be cured. Dienstag *et al*[28] reported that the fibrosis of cirrhosis was reversed during 3.5 years lamivudine treatment. Several reports also confirmed the improvement of fibrosis levels with anti-HBV drugs[29,30].

However, the regression of fibrosis occurs even in the natural course of CHB, where remission of inflammation usually follows the aggravation of hepatitis and progression of fibrosis that occurred during the HBeAg-positive stage[31]. In the past, several reports showed the improvement of fibrosis during the natural course of CHB. Fong *et al*[3] showed the improvement of histology comparing before and after the seroclearance of HBsAg. Waneless *et al*[32] reported that most cirrhotic livers showed some findings indicating the regression of fibrosis, irrespective of etiology. Natural regression of fibrosis consists of three steps, that is, thinning of fibrous septa, regeneration of hepatocytes and recovery of acinal structure. The regression of fibrosis occurs at a particularly early stage of improvement[6].

Recently, Jang *et al*[33] reported improvement of the long-term prognosis with anti-HBV drugs for patients with HBV-cirrhosis. However, suppression of HCC development was not observed in these cirrhotic patients, while it has been confirmed in patients with chronic hepatitis[8]. These results strongly suggest that liver cirrhosis still provides a strong risk for HCC, even after the eradication of virus and suppression of

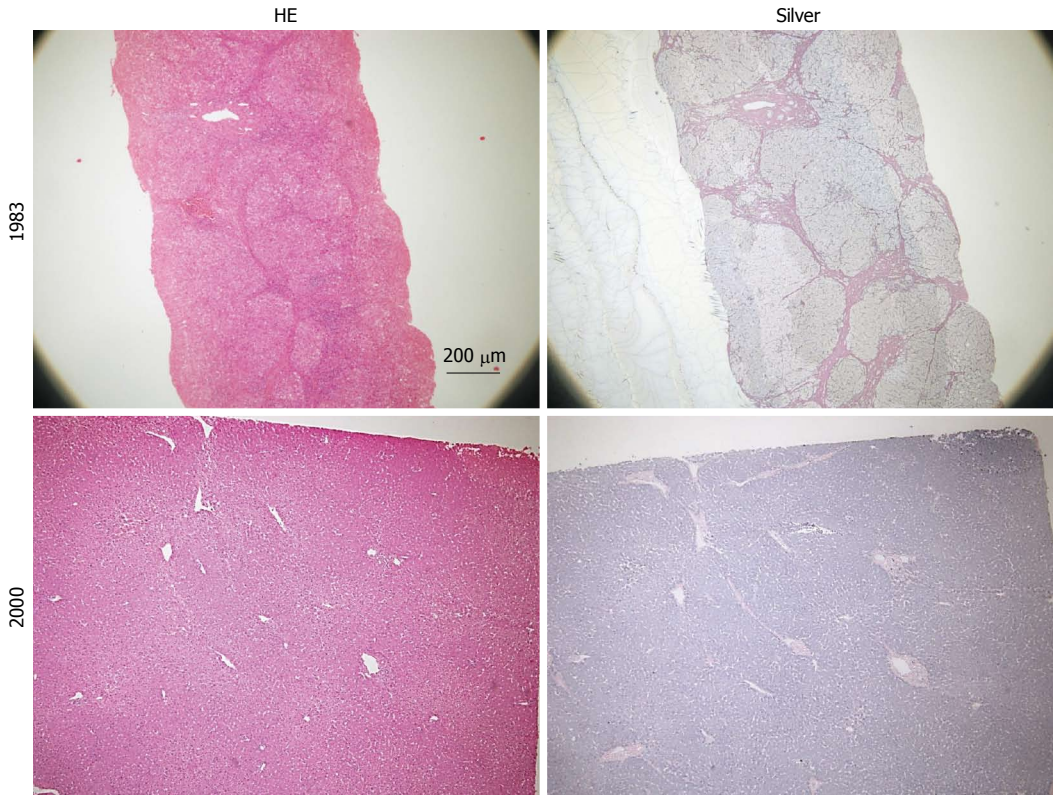

Figure 1 Liver histology of a patient obtained in 1983 (58 year old, needle biopsy) and 2000 (surgical resection of hepatocellular carcinoma). Slides stained with hematoxylin-eosin are shown on the left and those stained with silver are on the right. Marked reduction of fibrosis with improvement of inflammation is observed (This patient was patient 1 in our previous report[4]).

inflammation.

THE LESSONS FROM THE HCC CASES THAT OCCURRED AFTER HBSAG SEROCLEARANCE

We previously reported several cases with HCC that developed after seroconvesion from HBsAg to anti-HBs[4,34]. They progressed to liver cirrhosis or precirrhosis when they had HBeAg-positive chronic hepatitis several decades earlier and became inactive carriers with low viral load and long-standing normal transaminase levels after the seroconversion to anti-HBe. HCC developed further after the seroconversion to anti-HBs. Hepatic fibrosis was dramatically reversed and found to become only thin septa. Changes in liver histology of one patient[4] are shown in Figure 1. Similarly, Bortolotti *et al*[5] reported 2 pediatric patients with HBV-induced liver cirrhosis, whose fibrosis was almost completely resolved after normalization of transaminase levels. Similar to our cases, these results suggested that fibrosis of cirrhosis could be dramatically resolved when viral replication and inflammation were suppressed[31,35]. These cases, considering the dynamic natural course of HBV infection, give us some important suggestions about the clinical background characteristics of HBV-induced hepatocarcinogenesis.

First, as with anti-HBV treatment, these cases are typical examples showing that advanced fibrosis could be absorbed even in the natural course of CHB. After HBeAg-positive chronic hepatitis progresses to liver cirrhosis, the majority of patients follow the clinical course of long-standing remission with a low viral load. Considering this typical clinical course of CHB, natural regression of fibrosis might not be an exceptional phenomenon limited to these case reports, but it might occur commonly. This also suggests that some population of HBV-HCC patients with non-cirrhotic liver might have regressed fibrosis that was once cirrhotic or pre-cirrhotic. Consequently, our hypothesis is that the incidence and role of cirrhosis-derived HCC might be underestimated in HBV-HCC, and the roles of direct carcinogenesis due to HBV integration or viral gene function with a slight involvement of necroinflammation in non-cirrhotic HCC are overestimated.

HBV-cirrhosis is "macronodular" and has thinner septa than HCV[32]. Micronodular cirrhosis changes to macronodular with the suppression of inflammation[36]. This morphological observation might reflect the clinical course of these viruses, where HCV takes a continuous progressive course, and HBV is characterized by the alternating increases and normalization of transaminase levels.

Another important suggestion relates to the assess-

ment of fibrosis in CHB patients for the establishment of an effective HCC surveillance system. Recently, not only liver biopsy which is a gold standard for the evaluation of fibrosis, but a handful of methods to assess fibrosis levels have been used clinically. However, these methods may not discriminate these cases of "regressed fibrosis from cirrhosis", which might have more risk of HCC than "ongoing fibrosis". Thus, it might be possible to underestimate the risk of HCC in these patients with regressed fibrosis levels using these current methods. Indeed, needle biopsy might give the diagnosis of an almost normal liver with completely regressed fibrosis[32].

Cirrhosis-HCC mechanisms can be glimpsed in studies that examined the incidence of HCC after the seroclearance of HBsAg, which normally has a good prognosis[10,37,38]. Yuen *et al*[39] reported that the risk factors of HCC at this stage were the presence of liver cirrhosis and old age. Liver cirrhosis was also counted as a significant factor for the seroclearance of HBsAg[40]. This indicates that the strong immune reaction that caused liver cirrhosis eventually contributed to the seroclearance of HBsAg, providing some risk of HCC.

Similar suggestions could be applied to HCC cases with occult HBV infection (OHB). Some patients might have overt chronic HBV infection before entering the stage of OHB[41]. These patients might have "regressed fibrosis from advanced fibrosis" during the process of becoming seronegative for HBsAg. They can easily be missed during clinical surveillance of HCC, unless they were recognized as HBV carriers, possibly in early life[15]. Integration of HBV DNA or the direct action of an HBV gene such as HBx from long-standing viral replication is considered to play a role in the occurrence of HCC with OHB[42]. However, cirrhosis-HCC mechanism might play a central role in more number of patients with OHB-HCC than generally considered.

Liver biopsy has been a gold standard of evaluation for liver fibrosis[43]. Non-invasive methods for evaluation of fibrosis are widely used in clinical settings. They have been primarily used for the assessment of HCV-related fibrosis, and their application to HBV-induced fibrosis has always been under constant debate[44]. Transient elastography (TE) is one of the most reliable recent modalities to assess the fibrosis level without biopsy[45]. Several reports evaluated the efficacy of TE in both CHB and CHC patients and reported that diagnostic accuracy was almost equal between them[46,47]. However, a lower liver stiffness (LS) cut-off in CHB than in CHC in the advanced fibrosis stage was reported[48]. The interpretation of this was that fibrous septa were thinner in CHB than in CHC. Moreover, because CHB tended to have macronodular cirrhosis, TE waves go through liver parenchyma and produce a low stiffness value[49]. Likewise, Castéra *et al*[50] reported that the cut off values of TE in cirrhosis were higher in HCV-LC than that of HBV-LC and this difference came from the high prevalence of macronodular LC in HBV. Thus, although fibrosis markers are considered to be useful in the evaluation of fibrosis levels of CHB in general[49], there have been no studies attempting to assess the fibrosis levels following CHB patients from aggravation of hepatitis to the inactive stage.

APPLICATION OF MOLECULAR ANALYSIS TO THE "HCC FROM REGRESSED FIBROSIS"

Innovative advances of molecular analysis recently allow comprehensive cancer genome analysis in a short time, resulting in the accumulation of an overwhelming body of information about the cancer genome[51]. Schulze *et al*[52] analyzed the whole exon of 243 HCC tissues and reported that genomic pathways centered on three signaling abnormalities, β-catenin (CTNNB1), TP53, and AXIN1-related. They also suggested that they can distinguish HCC that arose from cirrhosis from those derived from non-cirrhosis using the difference in incidence of CTNNB1 TP53, a mutation of telomerase reverse transcriptase (TERT). In addition, mutations of TERT promoter have been noted as a characteristic genomic change of HBV-HCC, in which integration of the HBV genome frequently occurs[53,54]. It is interesting to know whether HCC that arose from "liver with regressed fibrosis from cirrhosis" is close to a cirrhotic or a non-cirrhotic pattern.

Telomere shortening is a general genetic marker of liver cirrhosis causing senescence of hepatocytes that is associated with the development of HCC[55-57]. Recently, TERT promotor mutations were observed already in premalignant nodules in cirrhotic liver[58]. In addition, Hartmann *et al*[59] reported that TERT gene mutations were frequently detected in liver cirrhosis irrespective of etiology, suggesting that they may be used to evaluate cancer-prone characteristics of liver. This result might be applicable in the evaluation of whether the liver with regressed fibrosis has oncogenic potential close to that of real cirrhosis, helping the understandings of the carcinogenic potential of that state. Such molecular markers that can assess cancer-prone characteristics of cirrhotic or non-cirrhotic liver are much needed to prove that a cirrhosis-HCC mechanism might be involved in the development of HCC than currently considered, even in low-fibrosis cases.

CONCLUSION

The clinical course of chronic HBV infection is dynamic and complex, with changes of serological markers, which is distinct from HCV infection that takes a more continuous clinical course. Because of this clinical profile, long-standing remission after established cirrhosis, result in the regression of fibrosis, where only thin septa are recognized. On the other hand, the integration of

the HBV genome or a direct gene function, such as HBx, may play an important role in so-called direct hepatocarcinogenesis. HCCs from regressed fibrosis and those *via* direct hepatocarcinogenesis might be categorized together into "HCC from non-cirrhotic liver". Thus, these mechanisms render the assessment of fibrosis as a risk factor for HCC very complex. Therefore, there is an urgent need to establish effective cancer surveillance system that addresses these complex issues. In addition, molecular markers to evaluate the risk of HCC in non-cirrhotic as well as cirrhotic liver should be clarified in a future analysis.

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TOPIC HIGHLIGHT

2016 Hepatitis B virus: Global view

X region mutations of hepatitis B virus related to clinical severity

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Abstract

Chronic hepatitis B virus (HBV) infection remains a major health problem, with more than 240 million people chronically infected worldwide and potentially 650000 deaths per year due to advanced liver diseases including liver cirrhosis and hepatocellular carcinoma (HCC). HBV-X protein (HBx) contributes to the biology and pathogenesis of HBV *via* stimulating virus replication or altering host gene expression related to HCC. The HBV X region contains only 465 bp encoding the 16.5 kDa HBx protein, which also contains several critical cis-elements such as enhancer Ⅱ, the core promoter and the microRNA-binding region. Thus, mutations in this region may affect not only the HBx open reading frame but also the overlapped cis-elements. Recently, several types of HBx mutations significantly associated with clinical severity have been described, although the functional mechanism in most of these cases remains unsolved. This review article will mainly focus on the HBx mutations proven to be significantly related to clinical severity *via* epidemiological studies.

Key words: Hepatitis B virus infection; Hepatitis B virus-X protein mutation; Hepatocellular carcinoma; Clinical severity

Core tip: Of hepatitis B virus (HBV)-X protein (HBx) mutations related to clinical severity, the A1762T/G1764A BCP mutation is one of the most frequently

encountered HBx mutations and plays a significant role in liver disease progression in chronic patients with HBV infections. It also further contributes to disease progression by inducing mutations of other HBx mutations related to clinical severity, such as G1386A/C (V5M/L), C1653T (H94Y), T1753V (I127V) and HBx C-terminal deletion or insertion. Moreover, T1753V (I127L,T,N,S) and C1653T (H94Y) mutations in the enhancer Ⅱ/BCP region and A1383C, G1386A/C (V5M/L) and C1485T (P38S) in the negative regulation domain are significantly related to severe types of liver diseases, including hepatocellular carcinoma. Furthermore, deletions or insertions affecting the C-terminal region of HBx may also contribute to the severity of the clinical outcome in chronic patients. In addition, our recent study indicated that a novel mutation type (X8Del) composed of an 8-bp deletion in the C-terminal region of the HBx could contribute to occult HBV infection in vaccinated Korean individuals *via* a reduced secretion of HBsAg and virions. Therefore, several distinct types of HBx mutations may contribute to HBV pathogenesis by regulating HBV replication or host genes related to cell homeostasis.

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INTRODUCTION

Chronic hepatitis B virus (HBV) infection remains a major health problem with more than 240 million people chronically infected worldwide, which potentially causes 650000 deaths per year due to advanced liver diseases including liver cirrhosis and hepatocellular carcinoma (HCC), particularly in endemic areas such as China and South Korea[1,2]. It is generally accepted that HBV infection accounts for approximately 50% of the HCC cases worldwide and even 80%-90% in highly endemic areas[1].

HBV is an enveloped Hepadnavirus belonging to the *Hepadnaviridae* family, with an incomplete double-stranded DNA genome of approximately 3.2 kb in length with four overlapping open reading frames (ORFs) encoding the polymerase (P), core (C), surface antigen (S), and X protein[3]. The S gene encodes a family of surface antigen polypeptides embedded within the viral envelope, which is a major target for diagnosis and protective vaccines. The C gene encodes the core antigen, which forms the nucleocapsid, within which reverse transcription of pre-genomic RNA occurs. The P gene encodes the virus reverse transcriptase, which also has RNase H and DNA polymerase activities[4-6]. Transcription of HBV proteins is controlled under four promoters (preS1, preS2, core and X) and two enhancers (EnhI and EnhII) in the viral genome, which overlap with those ORFs. Because it contains a polymerase without proofreading activity and uses an RNA intermediate (pgRNA) during its replication, the HBV genome has a higher mutation ratio than other DNA viruses[7-11]. Moreover, host immune pressures and interventions such as antiviral drugs and vaccines make the viral mutations more complicated[12-18].

Based on an intergroup divergence of > 8% in its complete genome sequence, the HBV strains are classified into eight genotypes, which are designated A-H, with a distinct ethnic and geographical distribution[1,19-21]. Different genotypes have distinct geographical distributions and usually induce various clinical outcomes. For instance, genotype C, the most prevalent genotype in Asia, is more prone to mutations and is associated with more severe liver diseases and lower antiviral responses compared with genotype B[3,22,23]. In particular, genotype C2 is reportedly responsible for the most chronic infections in South Korea. Indeed, several types of HBV mutations that are never or rarely encountered in other areas have been found in South Korea and have been proven through molecular epidemiologic or functional studies to be related to disease progression in chronic patients[24-44].

HBV X PROTEIN STRUCTURE AND FUNCTION

The HBV X protein (HBx) is a multifunctional non-structural protein that contributes to HBV biology and pathogenesis by stimulating virus replication or altering host gene expression related to HCC. HBx contains only 465 bp encoding the 16.5 kDa protein, which also contains several critical cis-elements such as EnhII, the core promoter and the microRNA-binding region[45-47] (Figure 1).

HBx plays a significant role in sustained HBV replication, which is a major risk factor for HCC development *via* proteasome inhibition[48,49], transactivation of HBV enhancer or promoters[50], autophagy induction[51,52], or polymerase activation by Ca^{2+}-dependent signaling[53-55]. HBx can also regulate HBV replication through epigenetic modifications, by being recruited onto the viral minichromosome in the nuclei of infected hepatocytes along with cellular histone acetyltransferases such as CREB-binding protein (CBP)/p300[56,57] and histone deacetylases such as HDAC1 and hSirt1[58]. HBx can help establish and maintain chronic infection by altering the patterns of host innate immunity, which causes the development and progression of chronic liver diseases in the absence of virus elimination[59,60]. HBx blocks apoptotic signaling and activates signaling pathways (such as NF-κB and PI3K) that override apoptotic signals from extrinsic ligands such as Fas or TNF-alpha[61,62]. HBx also plays an important role in hepatocarcinogenesis by inactivating the tumor suppressor p53[63], promoting Rb inactivation by

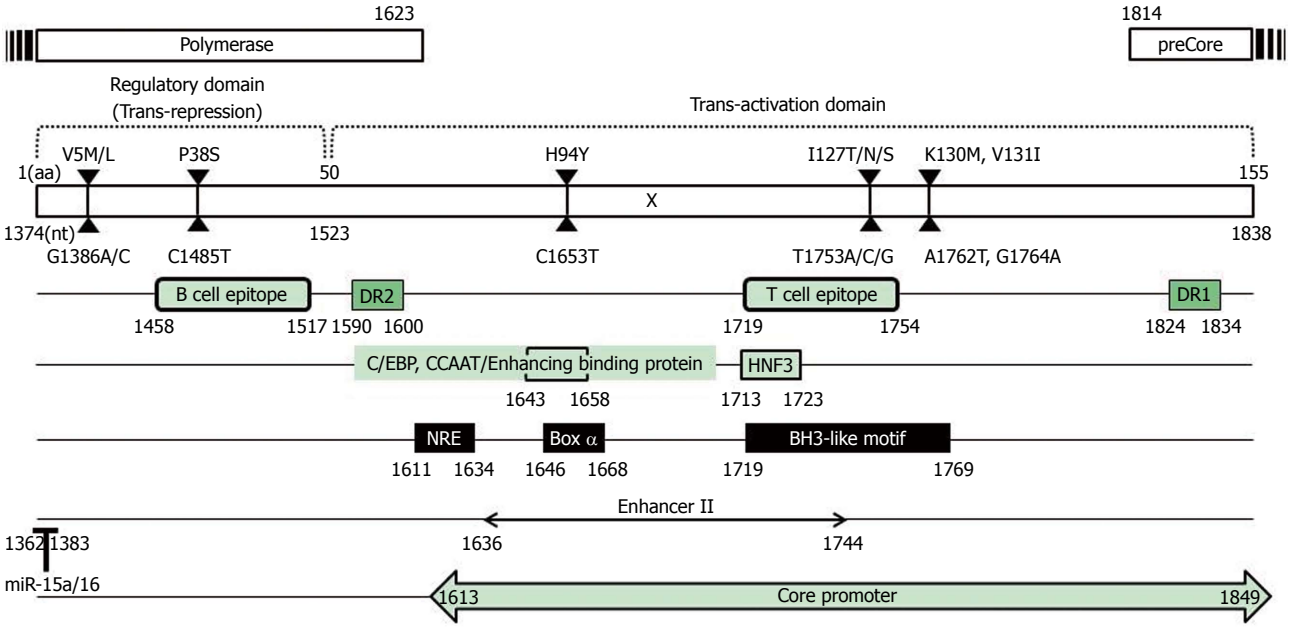

Figure 1 Hepatitis B virus-X protein genome structure. The HBV X region contains 465 bp (nt 1374 to 1838) encoding the 16.5 kDa HBx protein composed of 154 aa, which also contains several critical cis-elements such as EnhII (nt 1636 to 1744), the core promoter (nt 1613 to 1849) and the microRNA-binding region (nt 1362 to 1383). Thus, HBx mutations affected not only the HBx open reading frame but also the overlapped cis-elements. HBx: Hepatitis B virus-X protein.

phosphorylation[64], and compromising DNA repair mechanisms[65]. Consequently, mutations in the HBx ORF region may affect not only the HBx ORF and the overlapped cis-elements but also its binding capacity for host proteins. Recently, several types of HBx mutations significantly associated with clinical severity have been described mainly from chronic patients infected with genotype C[28,31,39,66-83], although the functional mechanism in most of these cases remains unsolved. This review article will mainly focus on the HBx mutations that have been proven to be significantly related to clinical severity *via* epidemiological studies (Table 1).

HBx MUTATIONS RELATED TO CLINICAL SEVERITY

Mutations in EnhII and (or) the core promoter region (BCP mutation, T1753V, and C1653T)

In general, 3 types of mutations in the EnhII/BCP region [one mutation in EnhII (H94Y: C→T of nt 1653) and two mutations in BCP (I127L,T,N,S: T→V of nt 1753, K130M and V131I: A→T of nt 1762 and G→A of nt 1764)] are mutational "hot spots", namely, the most frequently encountered among naturally occurring HBx mutations related to clinical severance from chronic hepatitis B patients, irrespective of genotype or geographical distributions (Table 1). The A1762T/G1764A BCP mutation leading to two overlapped HBx amino acid changes, K130M and V131I, is the most frequent HBV DNA mutation identified in many studies as being associated with HCC risk and outcomes[72,74,84-87]. The exact mechanism underlying the role of this mutation in hepatocarcinogenesis is still unknown. However, some underlying mechanisms have been recently elucidated. The mutation can cause a substantial decrease in HBeAg expression and enhancement of viral genome replication, which contribute to the liver disease progression *via* increased inflammation and viral invasion[88,89]. The mutation also leads to a truncated HBx protein, which not only promotes hepatocellular proliferation but also enhances HCC cell invasion and metastasis[90,91]. In particular, in chronic patients infected with genotype C2, this mutation is reported to be related to HBV genome deletion[26,31] or to be positively correlated with HBx M5V/L or H94Y mutations[28,37]. In addition, it may also contribute to hepatocarcinogenesis *via* reduced p21 expression, leading to rapid and uncontrolled cell proliferation[92]. A recent meta-analysis by Yang *et al*[82] revealed that the BCP mutation is present at significantly higher frequencies in HCC patients than in non-HCC controls, including patients with liver cirrhosis, chronic hepatitis and asymptomatic carriers. Our previous data using a Korean cohort with genotype C2-infected chronic patients also showed that the BCP mutation was the most frequently encountered mutation related to clinical severity (66.1%, 123/184 strains) and was significantly related to HCC [HCC (86.7%) *vs* chronic hepatitis (61%), $P = 0.017$; HCC (86.7%) *vs* asymptomatic carrier (24.4%), $P < 0.001$][28]. Our data also showed that during the natural course of HBV chronic infection, the most significant rise in the rate of the BCP mutation was found during the progression from asymptomatic carrier to chronic hepatitis (24.4%-61.0%), suggesting that the BCP mutation may play a major role in liver

Table 1 Mutations in the hepatitis B virus X region as related to clinical severity

Type of mutation	Mutations				Genotype	Clinical Significance			Region	Description	Ref.
	Amino acid		Nucleotide			HCC (%)	Non-HCC (%)	*P* value			
	aa	mutations	nt	mutations							
AS	4	NC	1383	A1383C	B/C	HCC (postoperative survival in patients with HBV-HCC) (P = 0.028)				Independent predictors of HCC survival	[65]
						52.8	25.8	0.003		miRNA binding site	[66]
AS	5	V5M/L	1386	G1386A/C	C	50.0	4.9	< 0.001			[27]
						47.8	0.0	< 0.001			[38]
						37.7	13.3	0.002			[67]
AS	30	NC	1461	G1461A/T/C	B/C	HCC (postoperative survival in patients with HBV-HCC) (P = 0.005)			B cell epitope	Independent predictors of HCC survival	[65]
AS	36	T36P/S/A	1479	A/G1479C/T/G	A/C/D	49.3	22.7	0.034	B cell epitope	B cell epitope	[66]
						15.3	7.8	< 0.001		B cell epitope	[66]
						80.0	17.1	< 0.010			[68]
AS	38	P38S	1485	C1485T	B/C	21.7	4.9	0.023	B cell epitope		[27]
						HCC (postoperative survival in patients with HBV-HCC) (P = 0.006)				Independent predictors of HCC survival	[65]
						48.7	13.9	0.001		Independent risk factor for the HCC	[69]
						29.9	16.8	0.001		B cell epitope	[66]
						30.4	15.0	0.038			[67]
AS	44	A44V	1504, 1505	C1504G, C1505G	A/D	35.9	6.9	0.012	B cell epitope		[70]
AS	50	G50R	1521	G1521A/C	A/D	60.0	4.3	< 0.010			[68]
AS	57	NC	1544	T1544A/C	B/C	HCC (postoperative survival in patients with HBV-HCC) (P = 0.039)				Independent predictors of HCC survival	[65]
AS	80	NC	1613	G1613A	B/C/C2	HCC (postoperative survival in patients with HBV-HCC) (P = 0.006)			Core promoter	Independent predictors of HCC survival	[65]
						54.7	28.3	0.001		Significance of association with HCC	[71]
						38.0	10.0	< 0.050			[72]
AS	81	NC	1613	G1613A	B/C	50.0	8.6	0.001	Core promoter		[73]
AS	86	NC	1631	C1631T	C	8.3	1.8	0.010	Core promoter	CP, NRE	[66]
AS	94	H94Y	1653	C1653T	B/C/C2	40.0	4.9	< 0.001	Box α, C/EBP,CCAAT/enhancing binding protein, Core promoter, EnhII		[27]
						HCC (postoperative survival in patients with HBV-HCC) (P = 0.015)				Independent predictors of HCC survival	[65]
						61.3	25.3	< 0.001		Significance of association with HCC	[71]
						56.0	30.0	0.0013			[74]
						45.0	19.0	< 0.050			[72]
						31.6	19.1	0.016		Box α	[75]
						35.4	18.6	< 0.001		Box α, CP, C/EBP, EnII	[66]
						41.2	13.3	< 0.001			[67]
						55.5	2.9	< 0.001			[73]
						8.9	2.2	0.017			[76]
AS	101	S101Stop	1675	C1675A	B/C	35.3	5.3	0.001	Core promoter, EnhII	EnhII	[77]
AS	106	S106T	1689	T1689A	C2	19.3	4.4	< 0.001	Core promoter, EnhII		[76]

AS	116	L/V116V/L	1719	T/G1719G/T	B/C	HCC (postoperative survival in patients with HBV-HCC) (P = 0.020)			BH3-like motif, Core promoter, EnhII, NRE	Independent predictors of HCC survival	[65]
						82.6	57.6	0.010		BH3-like motif, CP, EnhII, HNF3, T cell epitope	[66]
AS	117	NC	1724	T1724C	B/C	41.1	2.6	0.000	BH3-like motif, Core promoter, EnhII, NRE	EnhII	[77]
AS	118	NC	1727	A1727G	D1	35.0	5.0	0.001	BH3-like motif, Core promoter, EnhII, NRE		[78]
AS	123	L123S	1741	T1741C	D1	30.0	7.5	0.006	BH3-like motif, Core promoter, EnhII, NRE		[78]
AS, DM	127	I127L/T/N/S	1753	T1753C/A	C	36.7	12.2	0.007	BH3-like motif, Core promoter, NRE		[27]
			1753	T1753C	B/C	HCC (postoperative survival in patients with HBV-HCC) (P = 0.047)				Independent predictors of HCC survival	[65]
			1752, 1753	A1752C + T1753A/C/G	D	52.2	20.0	0.033			[79]
			1753	T1753A/C/G	C2	50.7	16.0	< 0.001		Significance of association with HCC	[71]
			1753	T1753A/C/G	C2	50.0	24.0	0.001			[74]
			1753	T1753C/G	A/D	43.6	17.2	0.041			[70]
			1753	T1753A/C/G	B/C	30.9	17.6	0.006		EnhII/BCP	[75]
			1753	T1753A/C/G	C	29.0	5.0	< 0.001			[67]
AS	130	K130M	1762	A1762T	C2	94.7	74.7	0.001	BH3-like motif, Core promoter	Significance of association with HCC	[71]
					B/C	80.0	25.7	< 0.001			[73]
AS	131	V131I	1764	G1764A	C2	98.7	78.7	< 0.001	BH3-like motif, Core promoter	Significance of association with HCC	[71]
					B/C	95.0	31.4	< 0.001			[73]
AS, DM	134	NC	1773	C1773T	D1	95.0	52.5	0.000	Core promoter		[78]
			1773, 1775	C1773T + A1775G	D1	17.5	0.0	0.010			[78]
AS	143	C143R	1800	T1800C	C	3.5	0.3	0.008	Core promoter	CP	[66]
AS, DM	100, 102	NC	1673, 1679	C1673T + A1679G	D1	17.5	0.0	0.010	Core promoter		[78]
AS, TM	128, 131	NC + V131L	1757, 1764, 1766	G1757A, G1764C + C1766G	D1	37.5	12.5	0.010	Core promoter		[78]
AS, DM	130, 131	K130M + V131I	1762, 1764	A1762T + G1764A	C	86.7	24.4	< 0.001	Core promoter		[27]
					D	HCC (HBV-DNA ⩾ 5 log copies/mL) *vs* CLD (HBV-DNA < 5 log copies/mL) (P < 0.05)					[79]
					C2	91.0	73.0	0.0035			[74]
					A/D	62.5	14.3	0.034			[80]
					A/D	64.1	20.7	0.000			[70]
					B/C	71.1	55.7	0.009		EnhII/BCP	[75]
					B/C	64.0	50.8	< 0.00001			[81]
					A/D	44.9	20.7	< 0.001			[82]
					C	91.5	53.3	< 0.001			[67]
					C2	60.7	22.2	< 0.001			[76]
Del, Ins	129-154, 120-148, 115-149, 135-154, 137-151	Deletion	93-94 (4aa), 79-80 (2aa), 93-94 (4aa), 151-152 (3aa)	Insertion	C2	HCC + LC (7.6%) *vs* CH + C (1.5%) (P = 0.017)					[30]

AA: Amino acid; AS: Amino acid substitution; BCP: Basal core promoter; C: Carrier; CH: Chronic hepatitis; CLD: Chronic liver disease; CP: Core protein; Del: Deletion; DM: Double mutation; EnhII: Enhancer II; HCC: Hepatocellular carcinoma; HNF3: Hepatocyte nuclear factor 3; Ins: Insertion; LC: Liver cirrhosis; miRNA: MicroRNA; NC: No change; NRE: Negative regulatory element; TM: Triple mutation.

disease progression, especially in the progression from asymptomatic carrier to chronic hepatitis in chronic patients infected with genotype C2[28]. This finding has also been confirmed by a recent meta-analysis[82]. Yang *et al*[82] also demonstrated that HBV-infected patients with genotype C, including HCC patients, have a significantly higher risk of BCP mutation compared with those with genotype B, suggesting that the BCP mutation can increase the risk of HBV-related hepatocellular carcinoma, particularly in an HBV genotype C population.

An HBV genome transfection-based experiment indicated that the BCP mutation can reduce the synthesis of HBeAg and enhance viral replication. However, a meta-analysis and our previous report also showed that there is no significant difference in BCP mutation prevalence between HBeAg-positive and HBeAg-negative chronic HBV-infected patients[28,82], suggesting that BCP mutation may occur in the HBeAg-positive immune tolerance phase earlier than in the HBeAg-negative immune clearance phase, at least in chronic patients infected with genotype C2.

The other HCC-associated T1753V mutation (I127L,T,N,S: T→V of nt 1753) was also shown to affect HCC survival[93,94]. The mutations in the HBx protein, which include an I127L,T,N,S amino acid substitution, can change the HBx binding affinity to BCL2, thereby affecting HBx-induced apoptosis[95]. Our previous data using Korean HBV-infected patients with genotype C2 showed that the prevalence of this mutation was also significantly higher in chronic patients with severe liver disease, HCC or liver cirrhosis than in patients who had milder types of diseases, were carriers or had chronic hepatitis [HCC and LC (34.3%) *vs* chronic hepatitis and carrier (13.4%), $P < 0.001$][28]. The other study using chronic patients from India who had genotype A or D revealed that this mutation is also usually associated with advanced forms of liver disease and had an increased risk of HCC[69], suggesting that the T1753V mutation may play a significant role in liver disease progression. Our previous report showed that the T1753V mutation is significantly related to the BCP double mutation [patients with the BCP mutation (31.7%) *vs* patients without the BCP mutation (11.5%), $P = 0.003$], but not to HBeAg serostatus[28]. A recent multivariate survival analysis by Xie *et al*[66] showed that the T1753V mutation is an independent predictor of HCC survival.

The C1653T mutation, leading to a simultaneous H94Y amino acid change in HBx, is located in box α, which is a strong activation element of the EnhII/core promoter, can enhance the box α binding affinity and EnhII/core promoter activity[96,97]. Because many trans-regulated nuclear factors bind HBV at the 1653 site, this mutation can alter the binding affinity of these nuclear factors. The C1653T mutation has been recently reported to be a predictive factor for HCC in Japan[75,98] and is associated with fulminant hepatitis and the acute exacerbation of HCC[99,100]. A recent multivariate survival analysis by Xie *et al*[66] showed that the C1653T mutation together with the T1753V mutation is also an independent predictor of HCC survival. Furthermore, our previous report showed that the C1653T mutation is significantly related to advanced liver diseases in Korean patients with genotype C2 infections [patients with HCC or LC (36.3%) *vs* patients who have chronic hepatitis or are carriers (12.2%), $P < 0.001$]. It has been reported that the C1653T mutation, together with 1762T/1764A mutations, can reduce the pre-C mRNA level (to approximately 55%) and HBeAg secretion in a transient transfection system using Huh7 cells[101]. Our previous study also demonstrated that this mutation tended to be related to an HBeAg-negative serostatus ($P = 0.087$) and was significantly related to the BCP mutation [patients with the BCP mutation (35.0%) *vs* patients without the BCP mutation (6.6%), $P < 0.001$].

Mutation in the negative regulation domain of HBx (aa 1-50) (A1383C, G1386A/C-V5M/L, C1485T-P38S)

The A1383T synonymous mutation, which does not cause an amino acid change in the HBx protein, is located in the negative regulation domain of HBx (aa 1-50), and this mutation was first found to be associated with HCC in a Korean cohort[28]. In one clinical study using Chinese cohort mostly infected with genotype B and C, this mutation was also associated with a worse prognosis in patients after liver transplantation[66]. Recently, a comprehensive analysis study based on global data by Li *et al*[67] showed that A1383T is one of the HBx mutations identified as independent risk factors for genotype C HBV-related HCC. It has also been reported that tumor suppressor microRNA 15a/16 (miR-15a/16) can directly target the wildtype HBx RNA sequence (nt1362-1383), inducing Bcl-2 expression by acting as a sponge to bind and sequester endogenous miR-15a/16. Consequently, this mutation can lead to a reduced binding capacity of miR-15a/16 to the HBx protein[47], which can prevent the infected cell from apoptosis by altering critical cell signal pathways and thereby contributing to hepatocarcinogenesis.

The G1386A/C mutation leading to a simultaneous V5M/L amino acid change at codon 5 of the HBx protein was first introduced by our previous study using a Korean cohort with genotype C2 infections[28]. Our data showed that this mutation was significantly more frequently found in HCC patients than in patients in other disease groups. Notably, the prevalence of this mutation was abruptly increased in HCC patients rather than in liver cirrhosis patients during disease progression (HCC *vs* liver cirrhosis; 49.2% *vs* 25.6%, $P = 0.024$), strongly suggesting that this mutation is a genuine HCC-specific mutation that possibly plays a pivotal role in the progression from liver cirrhosis to HCC[28]. Recently, the combination of both BCP double

mutations and both types of the V5M mutation, V5M and V5L, has also been reported to increase the HCC risk by 5.34 times compared with the wild type by inducing a higher NF-κB activity in transformed cells[86]. Our previous report showed that this mutation is significantly related to an HBeAg-negative serostatus [HBeAg-negative patients (40%) *vs* HBeAg-positive patients (19.1%), $P = 0.004$], suggesting that it may be generated from the immune clearance phase[28]. This mutation was also significantly related to the BCP mutation [patients with the BCP mutation (36.6%) *vs* patients without the BCP mutation (9.2%), $P < 0.001$]. To date, its clinical relevance has not been introduced except for a Korean cohort with genotype C2 infections. It is tempting to speculate that this mutation may play a pivotal role in hepatocarcinogenesis during the HBeAg-negative immune clearance phase during the natural course of genotype C2 HBV infection.

The C1485T mutation, leading to simultaneous P38S in the HBx protein, were first introduced as an independent risk factor for HCC development in a study by Muroyama *et al*[70] using a Japanese cohort with genotype C infections. Both studies using Korean cohorts with genotype C2 infections[28,68] and a recent investigation based on global data by Li *et al*[67] also revealed that this mutation is significantly related to HCC. A functional study supporting the relationship between the mutations with HCC still remains to be conducted. However, given that its mutation site is located at the B cell epitope region (Figure 1), this mutation may lead to persistent infection by providing a mechanism of evading the humoral immune response of the host.

Deletions or insertions in the C-terminal region of HBx

The C-terminal region of HBx plays a key role in controlling cell proliferation, viability, and transformation[102-105]. Therefore, C-terminally deleted or inserted HBx has reduced transactivation activity and inhibitory effects on cell proliferation and thus may contribute to HCC generation[106]. Moreover, its reduced transacting capacity might reduce HBV replication[107]. The C-terminal deletion or insertion is one of the most frequently reported mutations of HBx and has been frequently detected in tissues and serum samples from HCC patients, irrespective of genotype or geographical distribution[24,108,109]. Our previous report using a Korean cohort with genotype C2 infections showed that the prevalence of deletions or insertions was significantly higher in patients with severe liver disease, HCC, or cirrhosis of the liver (7.2%, 10/132), compared with patients who were carriers or had chronic hepatitis (1.5%, 2/135) ($P = 0.017$)[31]. All deletions in six strains were concentrated at the C-terminal end of HBx, encompassing the 113th to 154th codons. Four types of insertions (PKLL, GM, FFN, and tt) were observed in six patients. Notably, all insertions were accompanied by a BCP double mutation[31] (Figure 2). Furthermore, we first introduced a novel HBxAg vaccine escape mutation, X8Del with an 8-bp deletion in the C-terminal region of the HBx gene from 6 vaccinated Korean subjects[38]. Our *in vitro* and *in vivo* studies showed that this mutation causes a reduced secretion of HBsAg and HBV virions compared with the wild type, suggesting that the X8Del mutation may contribute to occult HBV infection in vaccinated individuals *via* the reduced secretion of HBsAg and virions, possibly by compromising the transacting capacity of HBxAg[38].

Other HBx mutations related to clinical severity

Recently, Xie *et al*[66] have reported 8 HBx mutational sites identified as significant independent risk predictors of HCC survival: 1383, 1461, 1485, 1544, 1613, 1653, 1719, and 1753 from a Chinese cohort mostly infected with genotype B and C. Despite the fact that the G1461V mutation is located at the B cell epitope, it (as a synonymous mutation) did not cause any simultaneous amino acid change in the HBx protein. Its regulatory modification in host cell or virion replication remains to be solved. The T1544V mutation also did not cause an amino acid change in the HBx protein. The G1613A mutation in the core promoter region is also a synonymous mutation, and its relationships with HCC have been reported in other previous studies.

Mutations in the BH-3-like motif of HBx can interfere with its interaction with two other Bcl-2 family members (Bcl-2 and Bcl-xL, which are critical for HBx to increase the intracellular calcium concentration), playing a significant role in viral replication and cell death[110]. Previous studies have reported that several types of mutations in the BH-3-like motif, T/G1719G/T, T1724C, and T1741C, were also significantly related to HCC[66,67,79].

The T1800C mutation leading to a simultaneous C143R amino acid change in the HBx protein is a novel genotype C HCC risk mutation identified by the Li *et al*[67] study, based on global data. To date, the function of this mutation in HCC remains unclear. However, of note, a recent study regarding HBV integration sites in 88 Chinese HCC patients showed that almost 40% of the integrated HBV genomes were cleaved at approximately nt1800, suggesting a potential role of this site in carcinogenesis, given that HBV genome integration has long been considered an important factor in HCC development.

CONCLUSION

In conclusion, HBx mutations may affect not only the HBx ORF but also the overlapped cis-elements. Considering all the HBx mutations related to clinical severity, the A1762T/G1764A BCP mutation is one of the most frequently encountered HBx mutations and plays a significant role in liver disease progression in

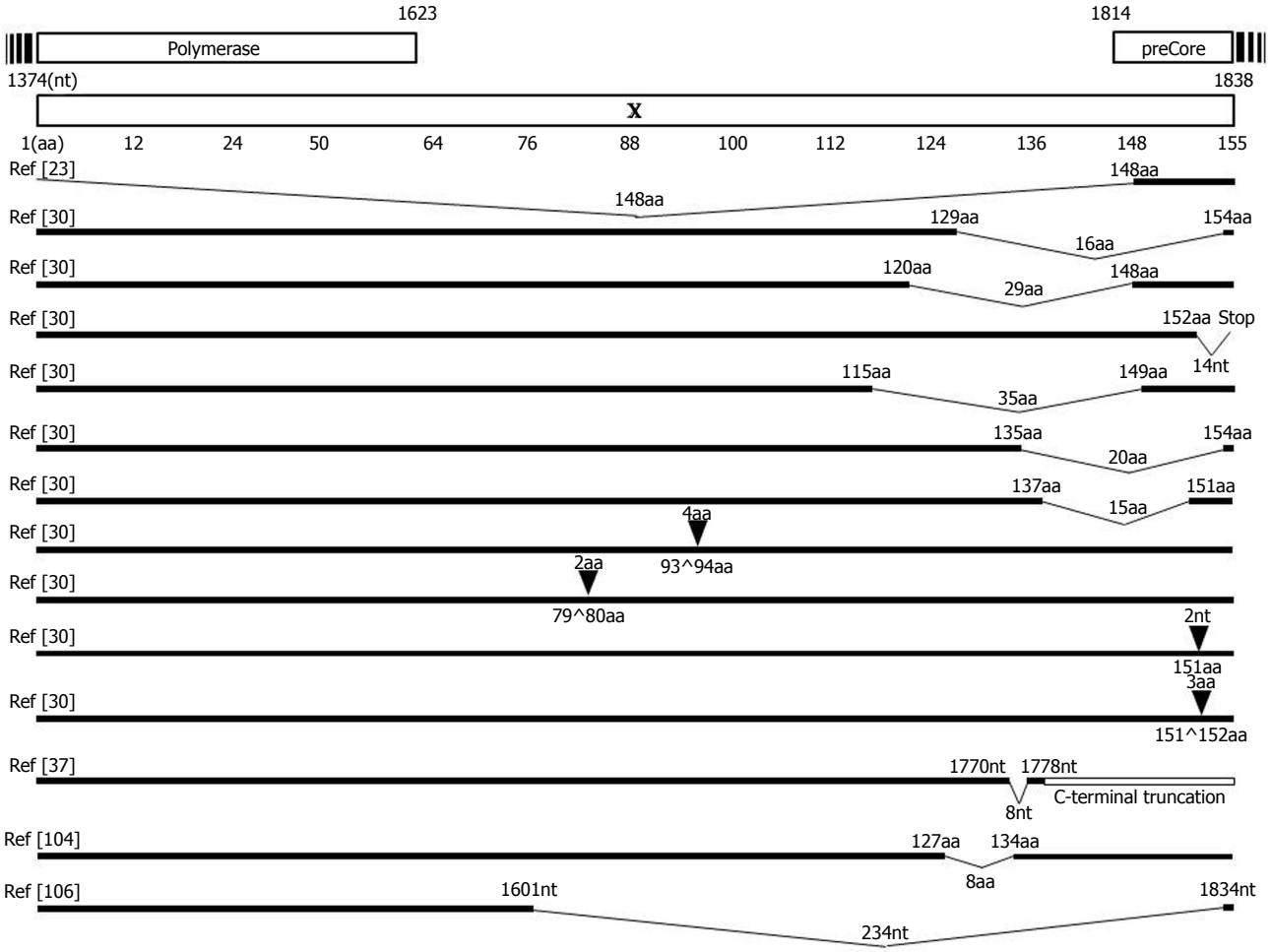

Figure 2 Mapping of deletions or insertions in the hepatitis B virus-X protein region. Deletions or insertions in the HBx region mainly occur in the C-terminal region of HBx, which may also contribute to the clinical outcome severity in chronic patients. HBx: Hepatitis B virus-X protein.

chronic patients with HBV infections. It also further contributes to disease progression by inducing mutations of other HBx mutations related to clinical severity, such as G1386A/C (V5M/L), C1653T (H94Y), T1753V (I127V) and HBx C terminal deletion or insertion. Moreover, T1753V (I127L,T,N,S) and C1653T (H94Y) mutations in the EnhII/BCP region and A1383C, G1386A/C (V5M/L) and C1485T (P38S) in the negative regulation domain were significantly related to severe types of liver diseases, including HCC. Furthermore, deletions or insertions affecting the C-terminal region of HBx can also contribute to the clinical outcome severity in chronic patients. In addition, our recent study indicated that a novel mutation type (X8Del) composed of an 8-bp deletion in the C-terminal region of the HBx contributes to occult HBV infection in vaccinated Korean individuals *via* a reduced secretion of HBsAg and virions. Thus, several distinct types of HBx mutations may contribute to HBV pathogenesis by regulating HBV replication or host genes related to cell homeostasis.

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REVIEW

Avoiding hepatic metastasis naturally: Lessons from the cotton top tamarin (*Saguinus oedipus*)

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Abstract

Much has been written about hepatic metastasis and animal models abound. In terms of the human experience, progress in treating this final common pathway, a terminal event of many human malignancies has been relatively slow. The current thinking is that primary prevention is best served by early detection of cancer and eradication of early stage cancers by screening. Some cancers spread early in their course and the role of screening may be limited. Until relatively recently there has not been a pathfinder model that makes the evasion of this unfortunate event a reality. This review discusses such an animal model and attempts to relate it to human disease in terms of intervention. Concrete proposals are also offered on how scientists may be able to intervene to prevent this deadly progression of the cancer process.

Key words: Cotton top tamarin; Hepatic metastasis; Carcinoembryonic antigen; Fibulin-5; Common marmoset

Core tip: Hepatic metastasis is a terminal event. Avoiding this complication would prolong life and current understanding of inflammatory mediators allows possible secondary intervention. The cotton top tamarin (CTT), like humans develops inflammatory bowel disease complicated by colorectal cancer but avoids liver metastasis. We suggest 5 mechanisms by which CTT avoid liver spread. They involve changes in ICAMs and their receptors, carcinoembryonic antigen (CEA) family mediators of angiogenesis, post translational modifications of molecules like CEA, and increased expression of anti-proliferative agents such as fibulins. This changes our perception from "monkey

see, monkey do" to "see what the monkeys do and do the same". Possible avenues of intervention are suggested.

Tobi M, Thomas P, Ezekwudo D. Avoiding hepatic metastasis naturally: Lessons from the cotton top tamarin (*Saguinus oedipus*). *World J Gastroenterol* 2016; 22(24): 5479-5494 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i24/5479.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i24.5479

INTRODUCTION

There are many animal models for human cancer and most of these do not occur naturally but are designed to mimic human disease by introduction of mutagen and may also employ promoters. Natural models lack this human "design" element but historically the differences may enhance understanding of disease causality and management for example, the Roux Sarcoma virus in the Plymouth Barred Rock fowl (chickens) and Bittner milk factor in murine mammary carcinoma among others. It is however unusual for a single animal to provide more than 1 natural model for disease[1].

Enter the cotton top tamarin (CTT), a pint-sized lower order, New World primate of the order Callitrichidae[2]. This remarkable monkey provides a model for human disease in inflammatory bowel disease and potential mechanisms[3,4], cancer of the lymphatic system[5], large bowel cancer[6], and immune-altered states[7], and recently liver metastases[8]. While a review of these models apart from the latter is beyond the scope of this review, it is significant that the example of the liver metastasis model could best be described as a "negative" or "avoidance model" in that the CTT with colon cancer avoids liver metastasis with an estimated frequency of 97.2%.

The hypotheses of how the CTT achieves this are open for discussion and debate has been ongoing since the first symposium, reported in 1985[9] where anatomical blood vessel variance was advanced as an explanation. This was refuted by veterinarians since the anatomy is similar to most mammals including humans (N. Clapp- personal communication). Our group has been exploring hypotheses based on adhesion molecules known to be important in human liver metastasis. While the article will focus on these molecular families in tamarins, it is important to first discuss our understanding of their importance in human "metastogenesis" as the findings in the CTT may enable intervention in the human where over 70% of GI cancer victims succumb to death caused by hepatic metastases.

Hypotheses of hepatic metastases in humans

Cancers of the colon and rectum comprise 14.5% of all cancers diagnosed in the United States (149880) and 11.1% of cancer deaths (51710) each year[10]. The primary treatment for large bowel cancer is surgery. Patients who recur do so largely in the liver, lung or peritoneum. While combination chemotherapy can increase survival even in advanced disease it is rarely long term. Metastatic colorectal cancer is therefore a major public health problem. Colorectal cancer is associated with chronic inflammation[11-13]. Inflammatory cytokines can be induced by interaction of terminally differentiated macrophages with the cancer associated glycoprotein carcinoembryonic antigen (CEA)[14]. CEA (CEACAM5) is a large heavily glycosylated glycoprotein of indeterminate function in the normal tissue in which it is expressed[15,16]. Discovered in 1965 by Gold and Freedman[17] it has become the most used tumor marker for colorectal cancers but failed to live up to the early expectations for use as an early detector of cancer[15,18]. It is a glycoprotein that is a member of a large gene family that consists of 29 genes divided into three subgroups that include the CEA-like glycoproteins and the pregnancy-specific glycoproteins (PSGs)[19]. These CEA like proteins are members of the much larger immunoglobulin supergene family[15]. The nomenclature for the entire CEA and PSG families is published in Beauchemin *et al*[20].

CEA

CEA is a GPI anchored glycoprotein with an average MW of 180 kDa this varies depending on the degree of glycosylation of the native protein. CEA contains 651 amino acids and these are found in 7 immunoglobulin like domains, an IgV domain at the N-terminus and six disulfide bridge linked IgC domains[15,16]. There are 29 possible sites for N-linked glycosylation and they tend to be of the complex tetra antennary type[15]. Recently a large number of functions in cancer cells have been attributed to CEA. These include a role in cell-cell adhesion[21,22], apoptosis (anoikis)[23], inflammatory responses leading to increased hepatic and possibly lung metastasis[14] and angiogenesis[24].

CEA AND ITS IMPACT ON LIVER METASTASIS: INDUCTION OF INFLAMMATORY RESPONSES IN THE LIVER

Liver metastasis is the major cause of death in patients with colorectal cancers. There is good evidence that cytokines play a major role in preparing the liver for implantation and subsequent growth of cancer cells[14]. It appears that a relationship exists between the colon cancer derived glycoprotein CEA and macrophage related cytokine production particularly associated with CEA Kupffer cell interactions in metastasis of colorectal

cancers to the liver. The discovery of CEA in 1965[17] marked a turning point in the study of cancer and led what might be called the age of tumor markers. CEA was the first commercially available tumor marker and may be considered the prototype[25]. Serum elevations of CEA (above 3 ng/mL) are seen in about 60% of colorectal cancer patients at presentation[26] and elevated levels are also seen in a number of other cancers including breast, gastric, lung and pancreas. Unfortunately, the early premise that CEA levels could be used as a screening test for colorectal cancer was not fulfilled. CEA was found not to be cancer specific and high false positive and false negative rates precluded its use in early detection of cancer. However, CEA is a useful marker in the determination of prognosis[27] and for detection of recurrence in the post-surgical follow up of colorectal cancer patients[28]. CEA has also been used for the detection of occult tumor by radioimmuno-detection[29] and as a target for treatment using radioimmuno-therapy[30]. In addition a number of studies have used CEA as the target antigen for immunotherapy[31]. Clinically CEA is very well understood, but its biological role is not clear and its function in the normal colonocyte still evades us. However over the years a number of functions mostly associated with tumor cell behavior have been attributed to CEA. We focused on the molecular interactions between CEA and its receptor CEAR in colorectal cancer cells and how disruption of these interactions and inhibition of cytokine production will affect the spread and growth of colorectal cancers. Designing an effective mimetic-based therapy requires the identification of the responsible molecules, mechanisms of action and regulation.

The most important functions of CEA are the effects associated with tumor cell survival whether it is at the primary site or at a distant metastasis. High levels of CEA are also associated with malignant ascites from colorectal cancer (CRC)[32]. CEA interacts with liver Kupffer cells through a cell surface receptor (CEAR) that we have identified as the heterogeneous RNA binding protein (hnRNP) M4[33]. The hnRNP group of proteins are multifunctional and are involved in many cellular events including alternative splicing, translational regulation and packaging of transcripts[34]. These proteins have also been implicated in the regulation of mRNA stability and translation in many cancers[35]. CEAR is a highly conserved protein that can bind both RNA and DNA and can transport mature RNA to the cytoplasm as well as acting as a splicing factor[36]. It can also be expressed both on the cell surface, in the cytoplasm and in the nucleus where it is most commonly found[37]. There are 4 isoforms of hnRNP M known[38]. Two of which we have shown bind CEA[33]. One form (hnRNP M4) which we originally identified as the CEA receptor has a 38 amino acid deletion between the first and second RNA binding domains. The longer form also binds CEA[33]. The two other forms have not been identified other than as proteins on 2D gels. CEAR will also bind to CEA in HT-29 colon cancer cells although the functional significance of this is not known. It has been suggested that it may be involved with the resistance afforded to anoikis[39,40]. Binding to CEAR occurs *via* a penta-peptide motif (PELPK) located at the hinge region between the CEA's N-terminal and first immunoglobulin loop domain. This interaction produces cytokines by activating a signaling cascade and these cytokines alter the liver microenvironment such that it becomes more hospitable to the implantation and growth of the cancer cells[41,42]. Production of both IL-6 and IL-10 by CEA stimulated Kupffer cells improves the survival of highly metastatic human colorectal cancer cells in the nude mouse intra-splenic injection model for liver metastasis[43] (see Figure 1).

CEA AS AN ADHESION MOLECULE

In 1989 Benchimol *et al*[22] demonstrated the first potential function for CEA. By transfecting tumor cells with the CEA gene they showed increased cell to cell adhesion and demonstrated that this could be inhibited using anti CEA antibodies. Adhesion was due to interactions between the N-terminal and the 5th and 6th (A3B3) immunoglobulin domains[44]. This suggested that these interactions may play a role in tumor cell behavior and in the development of metastases.

CEA AND ANOIKIS

Another important function that has been attributed to CEA expression is inhibition of apoptosis in particular the apoptosis caused by absence of attachment of cells to a substratum (anoikis)[23,40]. This would play an important role in metastases formation to a distant organ as unattached tumor cells in the circulation would be more susceptible to anoikis.

CEA AND ANGIOGENESIS

Here, we suggest a relationship between CEA, its receptor CEAR and angiogenesis. Others have also suggested a relationship between CEA and angiogenesis though they did not establish it as causal or identify potential mechanisms[45,46]. Recently, however they showed that secreted (soluble) CEA can directly activate endothelial cells *via* integrin β3 signaling[24,47]. We suggest that an alternate mechanism may also exist involving the relationship between CEA and its receptor CEAR. Interaction of CEAR with CEA in tumor associated stromal cells particularly macrophages is important. Disrupting that relationship may affect tumor growth and progression

It is significant that Low-Marchelli *et al*[48] have shown that CCL2 recruits macrophages to promote angiogenesis. We therefore suggest that colon cancer cells recruit macrophages by secreting MCP-1 and these macrophages are activated by tumor derived

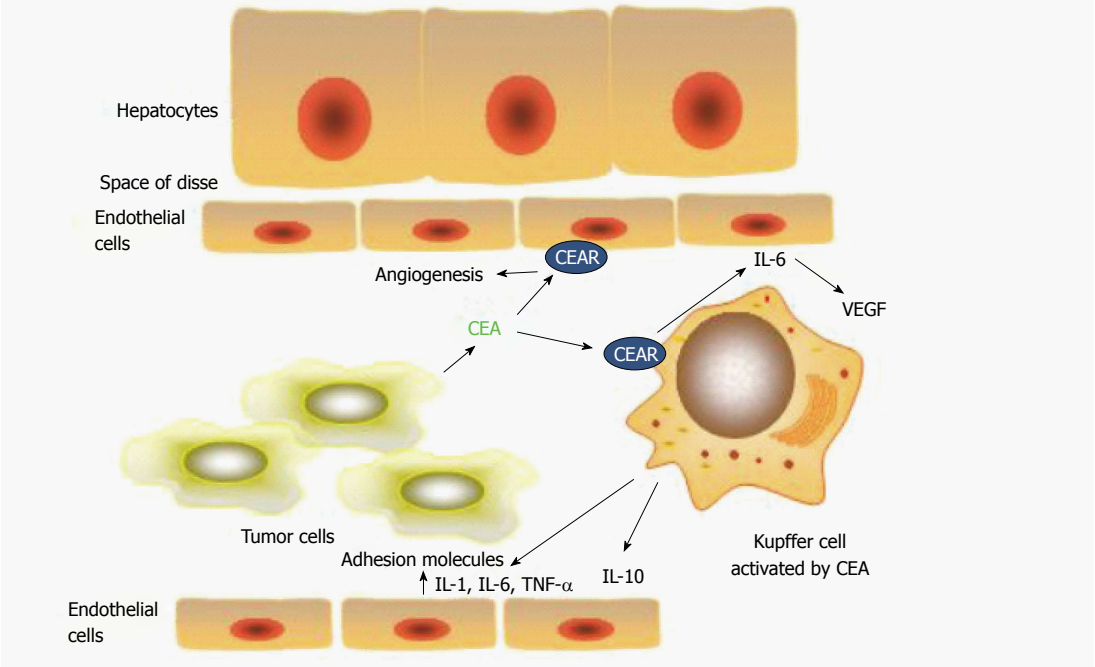

Figure 1 Schematic of the interactions of carcinoembryonic antigen in the hepatic sinusoid. Interactions of carcinoembryonic antigen (CEA) in the hepatic sinusoid. CEA released by the tumor cell binds with hnRNP M (CEAR) on the Kupffer cell surface resulting in release of the cytokines interleukin (IL)-1, IL-6, IL-10 and TNF-α. Effect of CEA induced cytokines on tumor cell interactions in the hepatic sinusoid. Cytokines IL-1, IL-6, IL-10 and TNF-α produced by Kupffer cells have a number of effects on the tumor cell microenvironment. These include up-regulation of adhesion molecules on hepatic sinusoidal endothelial cells. The most important of these seem to be E-selectin and ICAM-1. Cytokines such as IL-6 and IL-8 are pro-angiogenesis and they may also effect growth at the distant site[14,42].

CEA to secrete IL-6 and IL-8 both known pro-angiogenic factors[49]. Anti angiogenic factors are also produced (IL-10) and an imbalance between these two competing factions will tend towards pro-angiogenesis.

TARGETING CEA/CEAR INTERACTIONS AS A MEANS OF INHIBITING CYTOKINE PRODUCTION AS AN ANTI-METASTATIC THERAPY IN COLORECTAL CANCER

Because transformed cells home into tissues from the circulation in a highly selective way through complex molecular mechanisms, it provides a template for targeted therapy. Designing an effective mimetic-based therapy requires the identification of the responsible molecules and their mechanisms of action along with their regulation. The investigation of methods to block or modulate CEAR function *in vitro* and *in vivo* and relating this to tumor growth and development will increase our understanding of the molecular and biological mechanisms involved in the progression of gastrointestinal cancers. The investigation of methods to block or modulate CEAR function *in vitro* and *in vivo* and relating this to tumor growth and development has increased our understanding of the molecular and biological mechanisms involved in the progression of colorectal cancers. We have shown that the binding peptide YPELPK will induce cytokine responses in macrophages activated by CEA[50]. We have shown that the binding peptide YPELPK will induce cytokine responses in PMA activated THP-1 macrophages. These types of study are important for the development of rational therapies that will enhance those currently available.

OTHER POTENTIALLY PRO/ANTI-METASTATIC MOLECULES STUDIED

Fibulin-5

During the initial development of metastasis, the adhesion of tumor cells is mainly mediated through binding of extracellular matrix components to cell surface receptors[51]. Interestingly, one study[52] found an association of fibulin-5, the newest member of the fibulin family of extracellular matrix glycoproteins, with hepatic metastasis of colorectal carcinoma. Since fibulin-5 plays an important role in antagonizing angiogenesis[51-53] in humans, we sought to confirm the expression of fibulin-5 expression level in the CTT.

Avoidance hypotheses in the CTT

Our understanding of cancer and metastases in primates began when colon cancer was described in old world monkeys as early as 1914 and then in the new world in inter alia, Goeldi's monkey in the early 1960s. The latter, *Callimico goeldii* shares the Colombian habitat with the CTT and is a small black-furred New World lower order primate. At that time tens of thousands of CTT were imported by the pharmaceutical industry utilizing the "tamarin alarm reaction" to test the efficacy of anxiolytics although literature to document

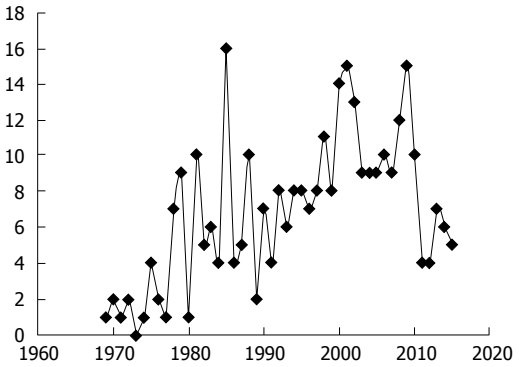

Figure 2 Graphic representation of published cotton top tamarin articles by year as estimated by a typical online search. The line chart is not necessarily inclusive of all cotton top tamarin (CTT) articles but merely confined to the search terms used. It is therefore used mainly to demonstrate the overall trends in annual publications devoted to this animal "CTT supermodel".

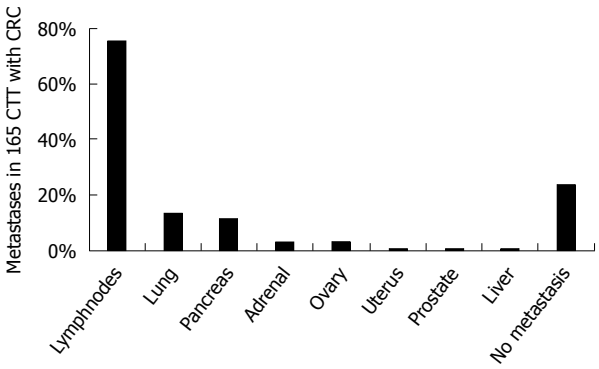

Figure 3 Schematic bar diagram depicting proportion of metastases in cotton top tamarin with colorectal cancer. The bar diagram shows the distribution of metastasis based on the ORAU colony cancer statistics. The paucity of liver metastases is remarkable. CTT: Cotton top tamarin; CRC: Colorectal cancer.

this is difficult to come by utilizing electronic search methods. Problems of CTT husbandry arose when the animals developed the "wasting marmoset syndrome"[54] postulated to be caused by a coronavirus but later effectively countered by improving the nutritional intake. The major challenge facing the CTT is the reduction of its numbers in its shrinking natural habitat in northwestern Colombia and most CTT are today found in zoos. As a result the species was listed as endangered in 1973 by the Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES)[55]. Since that time, the numbers of articles on the CTT had increased exponentially. This attests to the interest of the scientific community in the CTT specifically. Despite this interest, primate research in the United States is in decline with the closures of the ORAU (Oak Ridge Associated Universities) facility and that of the NEPC (New England Primate Center) which housed a significant proportion of CTT. The waning of public support and therefore funding opportunities combined with the expense of operations has largely been the cause of this decline. This is regrettable as it is generally not appreciated that monkey publication numbers are an extensive part of the scientific literature (678000 Medline™-listed publications) attesting to their usefulness and their command of the attention of the scientific community. Now, specifically CTT publications which were formerly about one tenth of non-human primate-themed publications (398 total CTT per National Library of Medicine ticket 28045-54653), are in decline in the example depicted graphically (Figure 2).

In view of the importance of this animal model applicable to many models of human disease, a CTT symposium was convened resulting in the publication of numerous articles in a supplement published in the journal Digestive Diseases and Sciences in 1985[9]. Thereafter a comprehensive book on the CTT was edited by Dr. Neil K. Clapp and published in 1993 by CRC Press[2]. This review seeks to build on that experience specifically focusing on the ability of the CTT to avoid hepatic metastases even though a large proportion of the animals develop advanced colorectal cancer. This is a unique feature of the CTT not typically found in other marmosets. It would appear that CTT peritoneal metastasis and ascites is also not common as it is with terminal human disease.

CTT MODEL FOR HEPATIC METASTASIS

The CTT develops colitis in the juvenile years and about one third (34.5%) go on to develop colon cancer and die of the disease. An observational study showed only 1.2% of animals had hepatic metastases[56] (Figure 3).

The etiology of the cancer seems to be the de novo inflammation-dysplasia-cancer variety although animals with adenomas have been described[56]. The risk of cancer deaths shows an increase from 3-8 years and then a gradual decline after age 12 similar to the recently described risk in humans with positive family history of CRC[57]. The etiology is still unknown but stress and micro-organisms may play a role[3,4]. Over half have multiple primaries diffusely distributed but the descending colon has the greatest proportion similar to the rectosigmoid colon predominance in ulcerative colitis cancer location.

CTT COLON CANCER BIOLOGY VIS A VIS HUMAN

Despite similarities in cancer biology between CTT and humans it can be seen from Table 1 that the anticipated mutations are either not described or characterization has not been attempted.

When considering changes associated with metastases[63] no CXC, CXCR4, CXCL12 (fusins), CCR7 (chemokine receptor 7) chemokines thought to be important in metastases have been described as yet in the CTT. Interestingly CXCL12 has been described in the common marmoset[64] and thus likely to be expressed in the CTT but is not necessarily a given.

Table 1 Comparison of cancer genetics in the cotton top tamarin and human

Stage of neoplasia	Sporadic human CRC	Human IBD-associated CRC	CTT colitis-CRC
Normal-appearing mucosa	*APC* initiator	Inflammation leading to mutations listed below	No cell line mutations in APC exon 4 and 15[58]
Early adenoma/indefinite dysplasia	Aneuploidy, methylation, Sialyl Tn	Aneuploidy, methylation, Sialyl Tn, MSI	90%diploid; no methylation[59]
Early promotor	MSI; *kRAS*, COX-2	DCC	kRAS absent[60]
Intermediate adenoma/low-grade dysplasia	c-src	c-src	c-src ND
Intermediate promoter	DCC/DPC4	*kRAS*	DCC/DPC4 ND; kRAS absent[60]
Late promoter	p53	*APC*	p53 ND; as above for APC exon 4 and 15[58]

Adapted from Itzkowitz and Harpaz 2004[61]; and Mattar MC, Lough D, Pishvaian MJ, Charabaty A[62]. APC: Adenomatous polyposis coli gene; MSI: Microsatellite instability; DCC: Deleted in colon cancer; *kRAS*: V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; COX-2: Cyclooxygenase 2 gene; c-src: Rous sarcoma gene that encodes for usual C-terminal inhibitory phosphorylation site (tyrosine-527); DPC4: Deleted in pancreatic carcinoma, locus 4; p53: Protein 53 kilodaltons; ND: Not done.

The human kallikrein zymogens (hK2) closely related to prostate specific antigen (PSA) have been studied in the CTT[65]. They concluded that the CTT has no functional hK2 or PSA. Since these moieties regulate cancer cell survival and growth the CTT may be a natural knock-out model for the study of this topic. We see in these findings yet another potential mechanism by which the CTT impedes cancer proliferation. Likely Toll-like receptors (TLR) also remain to be discovered in the CTT and may have likely similar functions to the human.

Since lectins[66] and adhesion molecules have been described in the CTT we have focused our attention on these molecules, particularly CEA. We also explored extracellular matrix proteins as the interaction between these proteins and cells play an important role in tumorigenesis and metastases. One such protein, fibulin-5[51-53] has calcium-binding EGF-like domains and associate with vascular and vascular structures. It is thought therefore to have anti-angiogenic actions and be an inhibitor of metastasis. It has not as yet been studied in the CTT.

CEA HOMOLOGUES IN THE CTT AND HUMAN

Cross reactive CEA species in monkeys were known when we published our seminal paper in 1994[67]. Haagensen *et al*[68] had reported CEA in higher order non-human primates, including the chimpanzee and gorilla. A mouse analogue had also been described by Abraham Fuks' group in 1989[69]. Our initial experiments used a number of antibodies to CEA including the T84.66 antibody developed by Dr. J. Shively's laboratory group at the Beckman Institute at the City of Hope which was used in the CEA kit commercialized by Roche™. This antibody when used in immunohistochemistry produced consistently negative results when using colon CTT fixed tissues and later native antigen in CTT colon tissue extracts in early experiments[70]. We tested other antigens such as organ specific neoantigen[71], thought to be the mediator of the lymphocyte adherence inhibition test for cancer and found reactivity[72]. In the early 1990's Karel Kithier of the Department of Pathology at Wayne State University was beta-testing a CEA kit produced by Tosoh Medics (CEA-AIA PACK kit™) which seemed to have a greater proportion of positive results when compared to another CEA kit marketed by Abbot. These results were expanded and confirmed and found to yield similar levels in CTT and humans with IBD[60,73] which opened a pathway to possible intervention[33] in addition to other cancer related moieties such as telomerase[60]. In order to show the CEA molecular moieties recognized by the kit we obtained both solid phase and tracer antibodies from Hybritech Inc., from Dr. Harry Rittenhouse. The resultant Western blotting[67] showed a specific 50 kDa band shared by CTT and humans (Figure 4).

A control blot using T84.1EC monoclonal which reacts with both NCA and CEA did not recognize the 50 kDa shared antigen in the same samples. Based on the known epitopes on the antibodies concerned we were able to speculate that the shared antigen included the Ig-like loop domains however the exact structure of this moiety remains unknown. In this publication we did hypothesize that "the smaller CTT CEA molecule might lack the critical peptides necessary for uptake and hence explain the paucity of liver involvement in CTTs with CRC".

EXPRESSION OF THE CEA GENE FAMILY OF PROTEINS IN THE CTT AND HUMANS

The next question we needed to answer was if an entire repertoire of CEA-family adhesion molecules were expressed by the CTT and if this subspecies was unique in this respect in the *Callitrichidae* family. In a later paper in 2000[73] we explored these questions by expanding the repertoire of antigens and extending our observations to include a close cousin of the CTT, the common marmoset (Callithrix jacchus - CM) which is

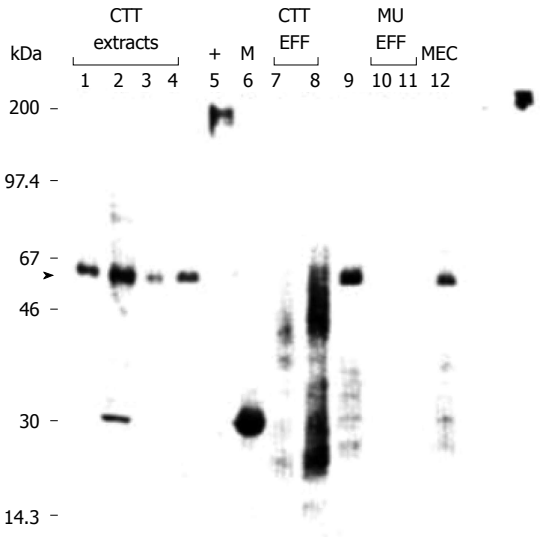

Figure 4 Specific carcinoembryonic antigen bands shared by cotton top tamarin and humans. Western blot using solid phase anti-CEA monoclonal antibody. Immunoblotting was performed using 5.6 pg protein/ml antibody after electrophoresis of a 12% SDS-polyacrylamide gel run under reducing conditions. Relative mobility (M) is shown on the left, and the type of samples loaded at 10 pg protein/lane are shown above the numbered lanes. Cotton-top tamarin extracts are in lanes 1 to 4 and effluent samples in 7 and 8. An extract from a patient with histologically proven rectal cancer and IBD is in lane 9, with human effluent samples in lanes I0 and 11 and human meconium in lane 12. Lane 5 contains the positive human CEA control, and the M, markers (M) are in lane 6. An arrow indicates the M, - 50000 band evident also in human extract (lane 5) likely a deglycosylated moiety. (Published with permission of Elsevier[67] and modified). CEA: Carcinoembryonic antigen.

not endangered but does develop colitis uncomplicated by cancer. Aware that the changes likely to influence CEA uptake were at the N-terminal end of the CEA molecule we used antibodies directed at epitope in that region, including the aforementioned Tosoh Medics™ kit and T84.66 antibody to quantify the CEA in colonic washings.

We found that the concentration of CEA in washings was sevenfold times higher than in washings from humans with IBD. T84.66 antibody was able to demonstrate a faint high MW band in a specimen of colonic washing from a CTT by immunoblot. In the Western blot using T84.66 we could now also demonstrate specific bands (immunohistochemistry had been consistently negative) in CTT washings and tissue extract of Mr $\geqslant$ 90 kDa which is the size of the non-glycosylated protein core of the CEA molecule[74]. CEA levels assayed by the Tosoh™ kit in CTT extracts were significantly higher than those from CM extracts ($P < 0.005$). Consistent with this finding was a high mean value in sera of CTT (134 ng/mL) compared to undetectable levels in the CM. NCA levels tended to be the lowest in the CTT as compared to humans and CM. Both animals' samples were low when reacted with a CEA-family antibody. In contrast binding with the anti-BGP was seen in both animals. We concluded that the similarity between human and tamarin native CEA is greater than previously believed and that it was likely that the N-terminal epitopes were conserved in the tamarins.

OTHER ANTIGENS SHARED BY CTT AND HUMANS

By the end of 2000 we published an expanded group of antibody assays involving 7 additional common epitopes representing most colonic cell types and blood group/carbohydrate antigens, some of them accepted tumor markers[60,70-76]. Mucin antigens, epidermal growth factor (EGFR) and telomerase were shared by CTT and humans but *k-ras* p21 and adenoma antigen (Adnab-9) were not. This supported the notion that carcinogenesis was likely de novo and did not progress through the adenoma-carcinoma sequence. Our finding of EGFR reactivity in the CTT provided a basis[76] for fibulin-5 role in the anti-metastatic armamentarium and OSN (by reactivity of BAC 18 monoclonal) suggesting similarities in anti-cancer immune responsiveness *via* the lymphocyte migration reaction. We were able to demonstrate also significant levels of EGFR epitope in the urine of 5 CTT (0.152 ± 0.053 absorbance OD-background at 405 μm) by ELISA. CEA reactivity with various antibodies in these samples were low (mean < 0.05). Blood Group Substances (BGS) were detectable [Span 0.063; FBB 0.103; CaCo 0.054) as were Bac18.1 (anti-OSN antibody) at 0.06]. Wild-type (Table 1) Ras p21; src, common membrane antigen (CMA), were negative corresponding to the data derived from CTT tissue. The only pieces of the puzzle missing were the questions of CEA molecular homology and how to tie the available data together to explain how the CTT dodges liver metastases.

DEFINITIVE STUDIES ON THE HOMOLOGY OF THE CEA MOLECULE

A comparative study of the C-terminus of the CEA molecule by the Stanners' group looked at multiple primate and prosimian species. The membrane linkage in CTT and other monkeys was found to be GPI anchorage rather than transmembrane (TM) seen in mice and rats[77]. This paper showed that the more versatile glycophosphatidyl inositol (GPI) linkage with a greater functionality constellation likely confers a tendency to tumorigenesis. Mutational packages evolved over time, one applicable to most primates including humans, and the second confined to the Cebidae radiation of New World monkeys leading to inhibition of cell differentiation. The rate of Ka (indicator of selective pressure acting on a protein-coding gene) nonsynonymous mutations for this radiation is 7X higher than the average Ka in primates. This led us to postulate that if such mutations occur at the C-terminus, it is quite feasible that similar mutations occur at the N-terminus where they may affect uptake of CEA, inhibiting hepatic metastases. Having

Table 2 Representative somatic sequences from the PELPK region of carcinoembryonic antigen in humans, cotton top tamarin and common marmoset

HCEA-A1 PELPKPSISSNNSKPVEDKDAVAFTCEPETQDA
CTT-WT PELPKPSISSNNSKPVEDKDAGAFTCEPETQDA
CM-WT PELPKPSISSINSKPVEDKDAGAFTCEPETQDA
M1-A1 (CTT) PE**V**PKPSISSNNSKP**GG**DKDAGAFT**W**EPETQDA
M2-A1 (CTT) PE**VS**KPSISSNNSKP**GG**DKDAGAFT**W**EPETQDA
M3-A1 (CTT) PE**VS**KPSISSNNSKP**V**GDKDAGAFT**W**EPETQDA
M4-A1 (CTT) PE**VS**KPSISSNNSKP**V**GDKDAGAFT**W**EPETQDA
M5-A1 (CTT) PE**VS**KP**Y**ISSNNS**N**PVEN**K**DAGAFT**W**EPETQDA
M6-A1 (CTT) PE**VS**KP**FI**FSNNSKP**GG**DKDAGAFT**W**EPETQDA
M8-A1 (CTT) PE**VS**KP**FI**F**R**NNSKP**GG**DKDAGAFT**W**EPGTQDA
M10-A1 (CTT) PELPKP**FI**F**S**NN**F**KPVEDKDA**V**AFTCEPETQDT
M14-A1 (CTT) PELPKP**FI**F**S**NN**F**KPVEDKDA**V**AFTCEPETQDA
M15-A1 (CTT) PELPKP**FI**F**S**NN**F**KPVEDKDA**V**AFTCEPETQDA
M16-A1 (CTT) PELPKPS**I**LSNNSKPVEDKDA**V**AFTCEPETQDA
M17-A1 (CTT) PELPKPS**I**P**S**NNSNPVEDKDA**VGL**TCEPDTQNT
M19-A1 (CM) PELPKPSIS**S**YNSKPVEDKDAGAFTCEPETQDA
M20-A1 (CM) PELPKPSIS**S**YNSKPVEDKDAGAFTCEPETQDA
M21-A1 (CM) PELPKP**FI**S**S**YN**F**KPVEDKDAGAFTCEPETQDA
M24-A1 (CM) PELPKPS**I**F**S**N**N**SKPVEDKDA**V**AFTCEPETQDA
M27-A1 (CM) PELPKPSISS**N**NSNPVEDKDA**V**AFTCEPETQDA
M31-A1 (CM) PE**V**SKP**FI**F**S**N**N**SKPV**G**DKDAGAFTCEPETQDA
M40-A1 (CM) P**G**VPKP**FI**F**R**IN**F**KPV**G**DKDAGAFTCEPETQDA

Sequences changes from those of wild type (germ line) are shown in bold. Published with permission of Springer[8].

also found common CEA-family and fibulin-5 ligand antigens we resolved to also investigate for parallel pathways of metastasis inhibition.

SUMMARY: SPECIFIC METASTASIS-TARGETED STUDIES IN CTT AND HUMANS

Using DNA extracted from the CTT tissues a Hot start PCR was performed and (1) sequences around the hinge region between the N-terminal and the first loop domain of CEA obtained using a Big Dye Terminator™ sequencing kit. We also immunolabeled human and CTT liver tissue using: (2) BGP (CEACAM1) antibody kindly supplied by Christophe Wagener (University of Freiberg, Germany); (3) CEAR (CEA receptor) polyclonal antibody; and (4) an antibody directed to fibulin-5 supplied by W. P. Schiemann (Department of Pharmacology, University of Colorado, Aurora, CO, United States). Finally, (5) we deglycosylated CTT CEA to determine the degree of N-glycosylation, as glycosylation may correlate with hepatic metastases. We thus examined 5 potential ways in which the CTT may evade hepatic metastases in 10 CTT and 25 humans.

To briefly summarize the observed[8] results (Table 2) we enumerate: (1) 63% of the CTT had non-synonymous PELPK pentapeptide mutations shown to be essential for hepatic uptake of numerous groups of protein including CEA and stromelysin, laminin, complement protein *etc.* CM had the PELPK mutations

Table 3 Biological attributes of the fibulin family[51-53]

Attribute	Fibulin-1	Fibulin-2	Fibulin-3	Fibulin-4	Fibulin-5
Invasion of endothelium	Decrease	Increase	Decrease	Unknown	Decrease
Binding of tropoelastin	Moderate	High	low	Moderate	High
Effect on angiogenesis	Unknown	Unknown	Reduced	Unknown	Reduce
MMP/ fibronectin change	Increase	Unknown	Reduced	Unknown	Reduced

to a lesser extent (73% *vs* 29%, $P < 0.05$). Sequence changes in the PELPK binding motif are found in the CTT that prevent binding to CEAR and do not allow activation of Kupffer cells and thus secretion of pro-inflammatory cytokines.

This could account for the high CEA serum levels in the CTT as compared to CM and humans and contribute to the lack of liver metastasis. Zimmer and Thomas in 2001 showed in a minority of patients with advanced colon cancer all of whom had high CEA levels, changes in the PELPK sequence. CEA from these patients could not bind to Kupffer cells. These patients did not have liver metastasis though peritoneal carcinomatosis was common[78]. Serendipitously: (2) Normal sections of human livers bearing metastatic disease (our intended "negative" controls) showed translocation of the BGP to the cytosol of the hepatocyte whereas CTT livers did not have detectable BGP except in the gallbladder wall. BGP is usually a structural protein found in the bile canaliculus but conceivably, if translocated to the cytosol, may act as a downstream mediator of VEGF, facilitating the progression of liver metastasis. Human cirrhotic and hepatitis livers demonstrated orthotopic BGP compared to human livers bearing metastases ($P < 0.02$) where the BGP had translocated to the cytoplasm (Figure 5).

This may answer the age-old clinical question as to why patients with cirrhosis rarely get hepatic metastases from extrahepatic primary cancers.We also observed: (3) a diminution of CEAR staining (Figure 6) in the CTT liver sections compared to humans ($P < 0.02$), a further barrier to CEA hepatic uptake in the CTT as compared to the human liver. Surprisingly: (4) Extremely robust fibulin-5 hepatocyte labeling was detected in CTT livers (Figure 7) and finally: (5) Minimal glycosylation of CTT CEA was demonstrated as opposed to human CEA. 80% of CTT *vs* 60% of CM were positive for fibulin-5 binding with functions of the various fibulins contrasted in Table 3.

Hepatocytes were the predominant cell types expressing fibulin-5 in both types of animals but tended to be more intense (Figure 6) in the CTT (0.75 ± 0.289 and 0.4 ± 0.418 respectively) and also seen to be of the same distribution and intensity in the human liver controls. Kupffer cells did not stain ($P = 0.033$ CTT and $P = 0.09$ CM) but occasional bile duct

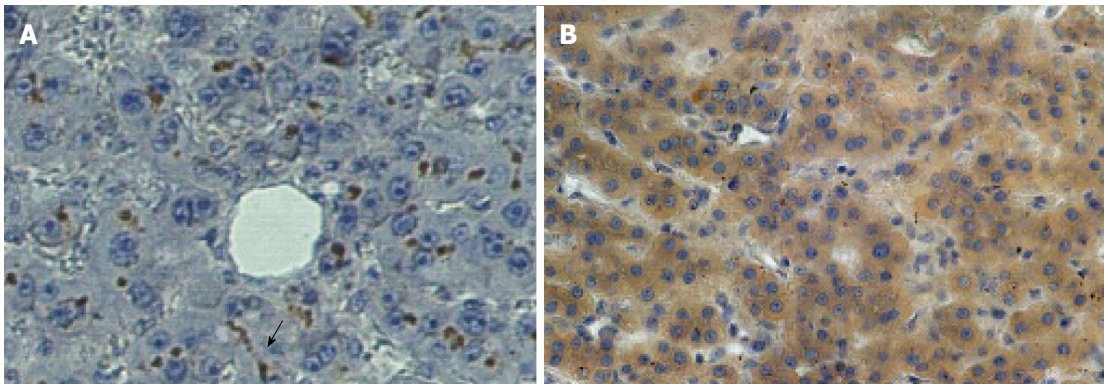

Figure 5 Distribution of CEACAM1 (BGP) in human liver. Photomicrographs of CEACAM1 staining with monoclonal antibody 4D1/C2 showing very intense brown staining mainly in the distribution of the biliary canaliculus in normal human liver (A).In contrast, in the normal portion of a liver from a patient with hepatic metastasis (B), dark staining is seen in the cytoplasm of the hepatocytes with no canalicular staining evident. Published with permission of Springer[8]. The arrow points to the typical distribution of bound ant-BGP in the bile canaliculus in the normal liver (A) at center, bottom.

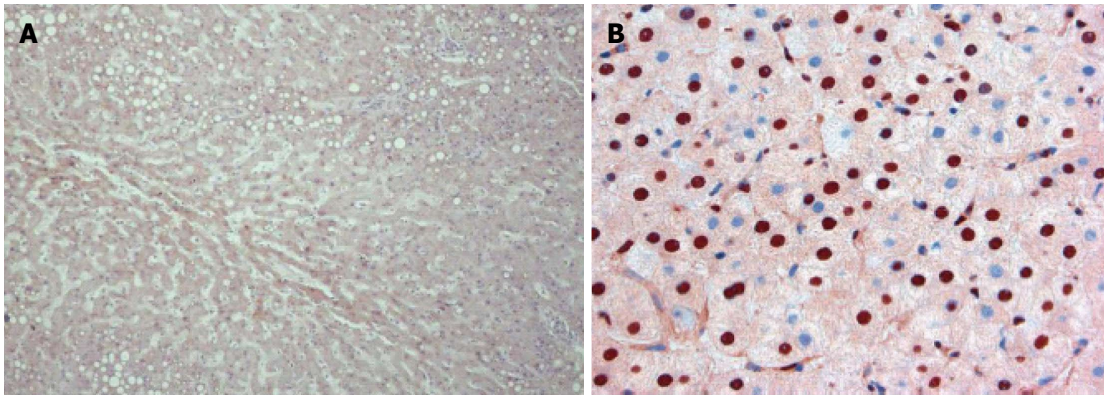

Figure 6 Distribution of carcinoembryonic antigen receptor in cotton top tamarin and human. The distribution of CEAR in the CTT (A) at low power can be seen in the cytoplasm of the hepatocyte by the light brown stain. In humans (B) at a higher power the increased intensity of staining in hepatocyte nuclei can be clearly seen. Published with permission of Springer[8]. CEA: Carcinoembryonic antigen; CTT: Cotton top tamarin; CEAR: Carcinoembryonic antigen receptor.

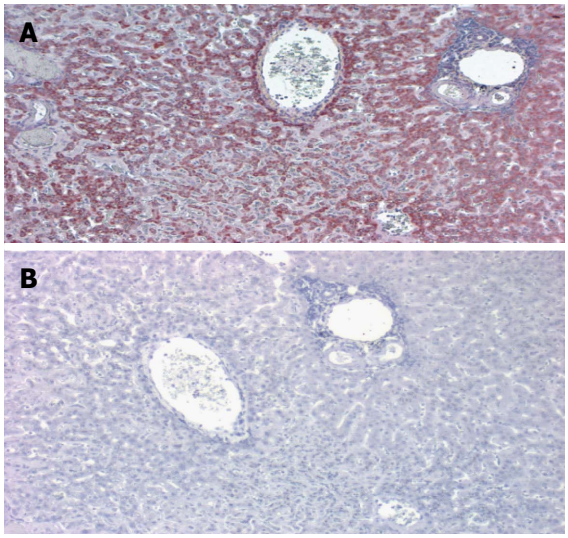

Figure 7 Immune labeling of CTT liver by anti fibulin-5 monoclonal antibody (A) and corresponding negative control (B). A: A central vein is seen in the upper center and a portal triad to the right. The dark red staining denotes the distribution of the antibody which is particularly intense surround the central vein. This is a low power magnification; B: In the negative control no staining is evident.

cells were positive but of lesser intensity in the CTT (0.125 ± 0.25 and 0.375 ± 0.479 respectively - Figure 8). Vascular staining was seen only in 2/5 CTT sections at a mean intensity of (0.3 ± 0.447). No appreciable uptake was noted in white blood cells in CTT and minimal in CM.

We therefore postulate 5 possible pathways of spontaneous hepatic metastasis evasion in the CTT, 4 of which we have previously reported[8], summarized in Table 4.

OTHER POTENTIAL ANTI-METASTATIC AND METASTOGENIC MOIETIES IN THE CTT

Lastly using the Gelco Diagnostics antibody kit to POA we determined a low level of POA[79] in extracts from CTT non-cancerous tissues 1.603 ± 1.656 μg/mL (mean ± SD). In contrast to serum in 13 humans with pancreatic cancer (9.622 ± 6.424) these levels were modest.Since normal serum levels in humans are < 15 U/mL even an order of magnitude increase in the

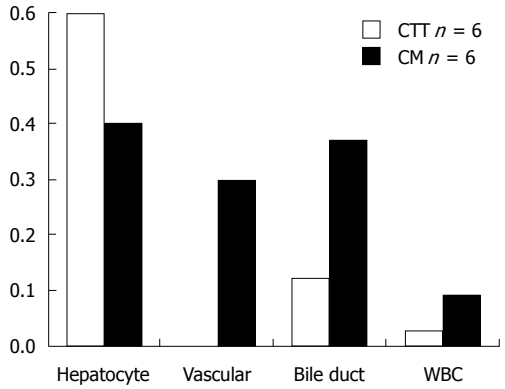

Figure 8 Summary of fibulin-5 immune labeling in cotton top tamarin, common marmoset and humans. Intensity of labeling: CTT and CM of Hepatocyte, Bile Ducts, Vascular Cells and White Blood Cells with Anti-fibulin-5. CTT: Cotton top tamarin; CM: Common marmoset.

CTT is unlikely to increase levels where they might influence the tumor invasiveness. In the study cited, a significant correlation was found between POA and CEA levels classified by stage of CRC (P = 0.003). Although further study is required it would appear that POA is not a major determinant in the CTT of the metastatic process to the extent that it is in humans making this another potential discouraging factor for the development of hepatic metastasis.

Ultimately, we would need to show that the cytokine environment milieu is similar in the CTT as described above for humans. Wilson *et al*[80] using 32 monoclonal antibodies has shown MHC Class 1 and Ⅱ proteins on lymphocytic B cells (CD20-21, 23) and CD16-56 on NK cells; CD2 and CD3 and CD4/8 helper T cells. In addition IL-2 receptor CD25 receptor is present as well as B chain LFA-1 CD 18. There was however poor reactivity for CD11a; and ICAM-1 (CD54) was negative. 14 years later with more reagents available, Kap *et al*[81] confirmed much of the above findings with IL1, 2, 4, 6, 10, 12, and 17a found. In addition they found all 4 clones against CD11a to be positive in contrast to the above paper. CD11a and CD18 and CD29 are important factors for adhesion and are expressed in the CTT. In addition, CD40 of the TNF family is present thus demonstrating that the CTT has almost all the necessary repertoire of proteins as is present in humans to develop hepatic metastasis. Although ICAM-1 was not included in the repertoire, by virtue of CTT colitis to respond to an anti-integrin monoclonal[82], we can conclude that selectins adhesion molecules are also likely expressed. This being the case, it would appear that the aforementioned inhibitory mechanisms are sufficient to abrogate this phenomenon almost completely.

We cannot rule out additional mechanisms and would encourage such research efforts. While ICAM1 may not have been confirmed in the above review (because it does not seem that it was included in the battery of monoclonals described above), E-selectin and integrins certainly have been convincingly demonstrated to be actionable by therapeutic intervention in the CTT[80-82].

Table 4 Summarization of our findings in this study

Parameter	CTT	CM	Human
PELPK Change	+ + + +	+ +	-
CEAR expression	±	±	+ + + +
CEA glycosylation	±	±	+ + + +
CEACAM1 expression	-	-	+ + + +
Fibulin-5 expression	+ + +	+ + +	+ +[1]

[1]Human data have limited staining data; Modified from and published with permission of Springer[8]. CTT: Cotton top tamarin; CM: Common marmoset; CEA: Carcinoembryonic antigen.

Certainly, the above hypotheses are not established scientific facts but they are testable and applicable to a particularly vexing problem presented by hepatic metastases. The ability to intervene using novel approaches based on these ideas will constitute the final section of this review and may represent an encouraging future vision of hope for myriads of "terminal" cancer patients.

In quoting the final chapter of the late Neal K. Clapp's book on this topic: "Our research opportunities using the cotton-top tamarins to study colonic diseases are truly only limited by our ingenuity".

HOW MIGHT WE EXPLOIT THESE ADVANCES FOR THE BETTERMENT OF THE CANCER PATIENT?

Hepatic metastasis of CRC is a very common clinical situation in oncology with prevalence at the time of diagnosis of approximately 20%-25%. The liver has been shown to be the most common site of metastatic spread of colorectal cancer. The prognosis of colorectal hepatic metastases has improved in the last few years owing to surgical resection of liver metastases for patients with no extrahepatic disease; however, liver metastases are resectable in only 15% of the cases[83,84]. Often, factors such as location, size and number of hepatic metastases make it difficult to resect these tumors[83,85]. Thus early prevention of metastasis is crucial in improving the prognosis of patients diagnosed with primary colorectal cancer. As earlier documented by Smedsrød *et al*[86] colorectal hepatic metastasis involve four steps which are interrelated; the cancer cells must establish micrometastasis in the liver by passing through microvasculature which is guarded by Kupffer acting in concert with NK cells. To ensure nutritional supply and establish clinically evident metastatic disease, these cells engage growth factors and adhesion molecules such as VEGF, VCAMs *etc.* As can be appreciated from both clinical and experimental evidence, Kupffer cells are involved with all the rate

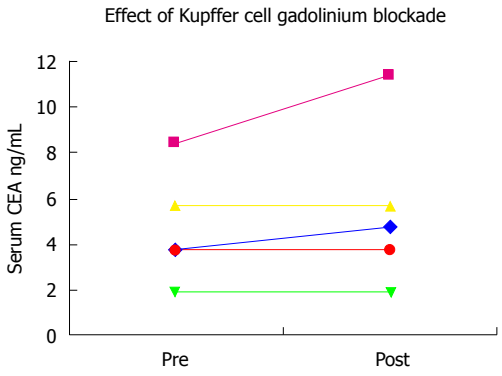

Figure 9 Carcinoembryonic antigen concentrations before and after gadolinium injection for magnetic resonance imaging. Five human patients had serial CEA levels measured before and after MRI with gadolinium contrast administration. No significant changes are seen after gadolinium for patients with normal or near-normal baseline levels. However the patient with a baseline CEA elevation showed a sizeable increase in the CEA level designated by the pink line. CEA: Carcinoembryonic antigen.

limiting step of this macrometastatic disease process which has been established in experimental systems but this role is not proven in human cancers[86]. These phases represent target points of recent therapeutic interventional designs.

USE OF GADOLINIUM BLOCKADE TO DEPLETE KUPFFER CELLS

In our earlier study using CTT[8], we showed that although they have CEA receptors similar to humans, they are able to evade CRC hepatic metastasis *via* two possible means; defective CEA receptors with much reduced receptor capacity or by sequence changes in the PELPK binding motif that is required for receptor activation (Table 2). We hypothesize exploiting methods to prevent CEA/CEAR binding in humans may prove effective in reducing the occurrence of hepatic and possibly lung metastasis. This hypothesis was tested earlier using gadolinium to block uptake of CEA by sinusoidal cells. Although the result was promising, only a transient blockage of CEA was achieved mostly due to the short wash out period for gadolinium (Figure 9). Therefore, using animal models this approach can be refined to optimize our earlier findings using gadolinium as blocking agent for Kupffer cells and the CEA/CEAR interaction.

As a novel approach to anti-metastatic therapy, gadolinium may be encapsulated in smart nano-devices that will prolong the residence time significantly. Using PEGylated-gellan-gum as the polymer of choice, gadolinium can be encapsulated in nanoparticles with average size of 250 nm[87].

Gellan gum is an exocellular microbial polysaccharide with a natural propensity to absorb biological fluid *in vivo* thus regulating the rate of drug/agent release. It consists of repeating tetrasaccharide units of glucose, glucuronic acid and rhamnose in a molar ratio of 2:1:1[88,89]. In addition to being used as a food additive, gellan gum has a wide range of applications in the pharmaceutical industry. In this area, its use has been mainly concentrated in ophthalmic drug delivery and oral sustained-release preparations, in which its ability to undergo cation-induced gelation is utilized as the main fabrication platform[87]. Gellan gum has a number of advantages as it can undergo temperature-dependent as well as cation-induced gelation, which may be of importance for *in vivo* slow release of encapsulated gadolinium. It also has proven non-toxicity in humans[90]. The FDA has also approved gellan gum as a food additive for human consumption[90], thus, its safety in humans is established. To improve the effectiveness of this targeting system, a three-dimensional matrix *via* covalent crosslinking with an end-reactive polyethylene glycol (PEG) through amide linkage can be used. Such modification provides a stronger gellan gum matrix that is nontoxic to normal cells and possesses the ability to deliver biological molecules such as gadolinium to target cells[91] in a controlled manner as illustrated in Figure 10. There is some experimental animal model work that suggests that gadolinium in the form of metallofullerenol nanoparticles inhibit cancer metastases[92] but the metastases observed were mainly pulmonary.

FIBULIN AS A TREATMENT FOR HEPATIC METASTASIS

The process of nanoprecipitation described by the schematics in Figure 10 can potentially be applied to protein encapsulation provided the formulation parameters are optimized as described earlier by our group[91]. Fibulin-5 plays an important role in antagonizing angiogenesis in humans; furthermore its overexpression has been shown to inhibit HCC cell migration and invasion *in vitro*[93], thus fibulin-5 could also be encapsulated in gellan gum nanoparticles to increase their *in vivo* residence time. Nanoparticles formulation should be optimized as described earlier[91] prior to encapsulation of fibulin-5. We would expect fibulin-5 to be the most effective of the fibulins in reducing angiogenesis but fibulin-3 may also be considered (Table 3). While fibulin-5 has been shown to suppress metastasis to the liver and lung[94], it has yet to be used directly in a nanotechnology formulation as we propose. A report that human oncogenic fibulin-5 may promote nasopharyngeal cancer metastasis[95] would suggest that we proceed cautiously with this approach with both agents.

TARGETING CEA/CEAR INTERACTIONS AS A THERAPEUTIC APPROACH TO LIVER METASTASIS

CEA has been shown to be a mediator of metastasis for colorectal cancer by causing changes in the tumor microenvironment that increase the potential for tumor

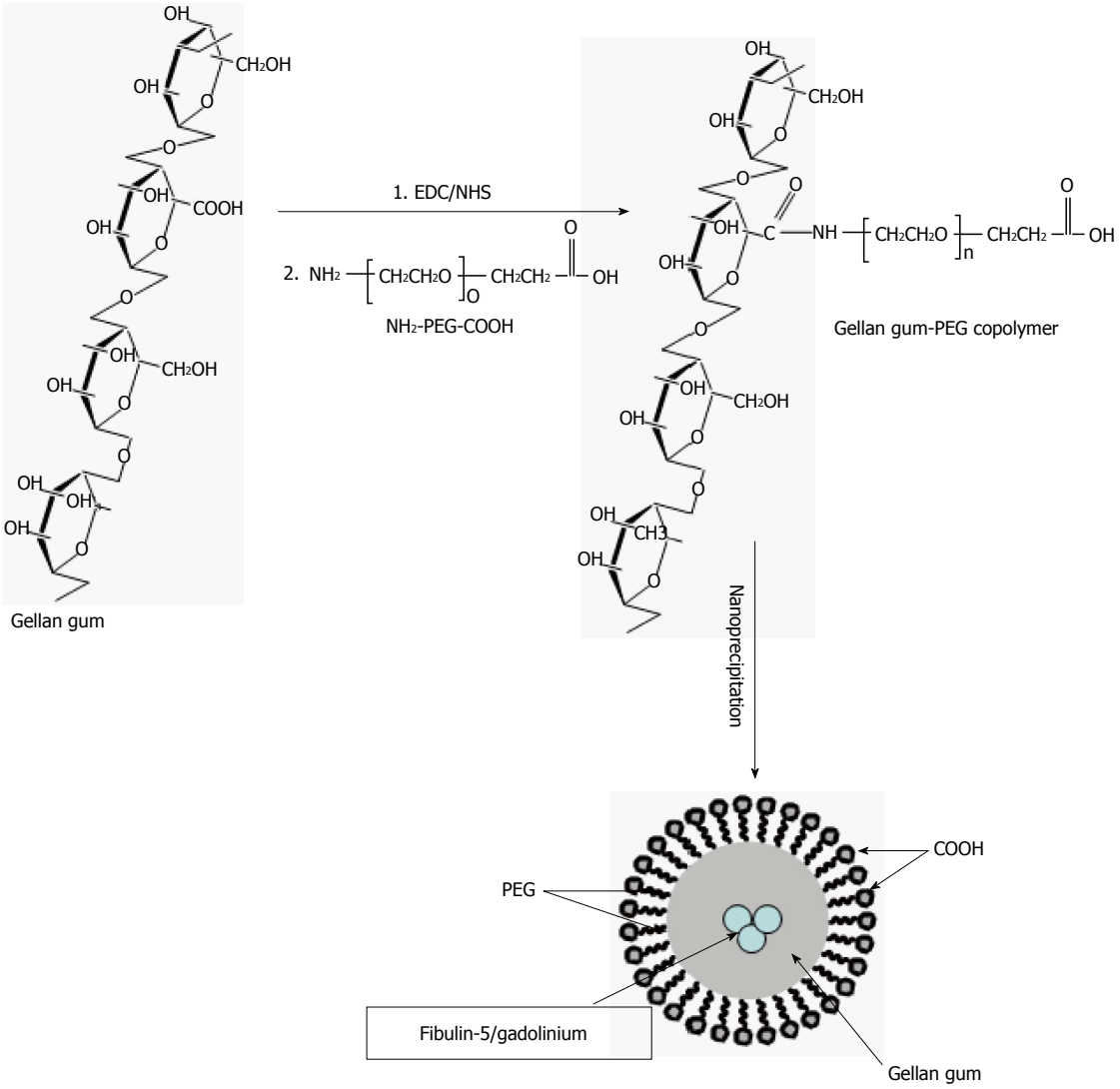

Figure 10 Schematic representation of the technique used for the synthesis of gadolinium encapsulated Gellan-gum-block-Polyethylene glycol co-polymeric nanoparticles. The above figure is a representation of the technical steps to generate nanoparticles of gadolinium encapsulated by Gellan-gum-block-Polyethylene glycol copolymeric micelle nanoparticles. This should ensure delivery and advantageous dwell time in order to block Kupffer functions of uptake of CEA and reduce the pro-metastogenic role of these cells. CEA: Carcinoembryonic antigen.

cell implantation and growth[14]. CEA interaction with its receptor therefore suggests a targeted approach to therapy. A number of methods could be used to achieve disruption of CEA/CEAR including the use of antibodies specific for CEA or its receptor. In general this type of approach has worked well against various cancers and growth receptors *e.g.*, EGFR. Other approaches using small molecule inhibitors of ligand receptor interactions are also being used, In particular RNA aptamers hold great promise as therapeutic agents[96]. These aptamers are now being used in studies involving CEA[97]. Lee *et al*[98] have described the use of a RNA aptamer directed against the PELPK motif of CEA to prevent liver metastasis from the mucinous colon cancer cell line LS174T. They suggest this occurred by disruption of CEA/CEAR interaction. LS174T produces large amounts of CEA and has a high metastatic potential to the liver[99]. Recently, aptamers have also been used against the homotypic adhesion sites of CEA to block tumor cell/cell interactions[100] further emphasizing the feasibility of this approach[101]. Aptamers are also being used for radiolocalization of CEA producing tumors and may even supplant the use of antibodies for this purpose[102]. The use of CEA antagonists to reduce the metastatic potential has now become feasible especially with the advent of small molecule inhibitors. More pre-clinical trials are needed before these approaches can be used in cancer patients at high risk for metastases.

CONCLUSION

We thus have the means to potentially interfere with the uptake of CEA from the hepatic sinusoid Kupffer cell receptor and hopefully disrupt the inflammatory cascade and well as intervening in the fibulin pathways in delivery of fibulin-5 to shore up hepatic defenses against tumor spread to the liver. In time, additional

interventions will be designed to make this a reality based on the lessons learned from the cotton top tamarin- the artful liver metastasis dodger.

ACKNOWLEDGMENTS

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REVIEW

Current status of intragastric balloon for obesity treatment

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Abstract

Endoscopic bariatric therapy may be a useful alternative to pharmacological treatment for obesity, and it provides greater efficacy with lower risks than do conventional surgical procedures. Among the various endoscopic treatments for obesity, the intragastric balloon is associated with significant efficacy in body weight reduction and relief of comorbid disease symptoms. Anatomically, this treatment is based on gastric space-occupying effects that increase the feeling of satiety and may also affect gut neuroendocrine signaling. The simplicity of the intragastric balloon procedure may account for its widespread role in obesity treatment and its applicability to various degrees of obesity. However, advances in device properties and procedural techniques are still needed in order to improve its safety and cost-effectiveness. Furthermore, verification of the physiological outcomes of intragastric balloon treatment and the clinical predictive factors for treatment responses should be considered. In this article, we discuss the types, efficacy, safety, and future directions of intragastric balloon treatment.

Key words: Intragastric balloon; Obesity; Bariatric; Metabolic; Endoscopy

Core tip: Obesity is a complex metabolic illness that is associated with several comorbid diseases. There has been a constant demand for safe and more effective weight reduction interventions to fill the gap in the treatment of obesity. The intragastric balloon is a fascinating intermediate alternative solution between

medical obesity treatment and bariatric surgical procedures for obese patients that may provide better efficacy and have a more favorable risk profile.

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INTRODUCTION

Obesity is a complex metabolic illness that results from excess accumulation of body fat and may lead to negative health consequences. Obesity increases the prevalence of various diseases, including diabetes mellitus, hypertension, coronary heart disease, sleep apnea, stroke, gastroesophageal reflux disease, gall bladder disease, certain types of malignancy, and non-alcoholic fatty liver disease[1]. Moreover, it is also a major avoidable health detriment. Current therapeutic approaches to obesity are lifestyle changes, pharmacologic treatment, and bariatric surgery. Although intensive lifestyle modification was reportedly associated with only limited weight reduction[2-4], when it is combined with weight-loss drugs approved for long-term use, an additional weight reduction of 3%-9% can occur within 1 year[5]. Such drugs are said to improve several cardiometabolic risk factors, but they are also related to harmful adverse effects[5]. Although new obesity medications have recently been approved and introduced[6-8], they are associated with issues of safety and high costs. Weight-loss surgery provides the most sustained and effective therapeutic choice for obesity. Accessible methods include the adjustable gastric band, Roux-en-Y gastric bypass, or sleeve gastrectomy[9,10]. Regardless of its proven effectiveness, only 1% of obese patients eligible for the surgical procedure choose to undergo it[11]. The major issues with surgery are difficult accessibility, high costs, patient non-preference, and significant morbidity and mortality. Although its associated mortality has decreased considerably, the complication rate in the early and late stages of the bariatric procedure persist at 17% (95%CI: 11%-23%)[10].

Therefore, minimally invasive and effective methods are needed for the treatment of obesity. As such, endoscopic bariatric treatment was recently introduced. It includes intragastric balloons, gastroplasty techniques, aspiration therapy, and gastrointestinal bypass sleeves. Among them, the intragastric balloon has been the most frequently used in practice and the most studied for obesity treatment.

INTRAGASTRIC BALLOON

In 1985, the Garren-Edwards gastric bubble (GEGB) was the first intragastric balloon approved for obesity treatment and was introduced in the United States market. It was made of polyurethane, had a cylindrical design, and was filled with 200-220 mL of air[12]. However, several adverse events were associated with its use, including small bowel obstruction associated with spontaneous deflation and gastric mucosal injury. Although the GEGB is no longer used, considerable advancements to its design have led to the development of a more effective and safer intragastric balloon. It is now being used in numerous countries. Additionally, the United States Food and Drug Administration (FDA) recently approved two new intragastric balloons.

The increased prevalence of obesity has motivated experts in bariatric medicine to advance minimally invasive endoscopic treatment for obesity management as well as innovative techniques that address important features of treatments, such as their efficiency and safety. A new meta-analysis showed that endoscopic obesity treatment could be effective and of substantial value if combined with a multidisciplinary and comprehensive treatment plan[13].

The intragastric balloon technique has become an effective method of achieving significant weight reduction in obese people (Figure 1). One or more intragastric balloons can be placed in the stomach using endoscopic procedures under mild sedation in an outpatient setting. Intragastric balloons allow patients to sense fullness and ultimately reduce their food intake. It is hypothesized that the intragastric balloon facilitates satiety peripherally by being an obstacle to food consumption, decreasing intragastric volume, and delaying gastric emptying[14]. Additionally, signals transmitted centrally through the vagal nerves by activated gastric stretching receptors could affect satiety[14]. The intragastric balloon permits an early feeling of satiety, which is thought to be a consequence of gastric distention. The mechanical intragastric distention to a meaningful volume during mealtime significantly decreases the amount of food eaten[15,16].

The intragastric balloon may also act *via* its relationship with various neurohumoral factors. It affects hunger control and gastric emptying by altering gut hormones and peptides such as ghrelin, leptin, cholecystokinin, and pancreatic polypeptide[17,18] and may also be related to physiological adaptation to weight loss.

The intragastric balloon may play diverse roles in obesity treatment as a preemptive therapy, a metabolic therapy, or a primary therapy.

TYPES OF INTRAGASTRIC BALLOONS

Orbera intragastric balloon

The most frequently used balloon is the Orbera Intragastric Balloon (Apollo Endosurgery, Austin, TX, United States), which was known previously as the BioEnterics Intragastric Balloon (BIB). It is an elastic

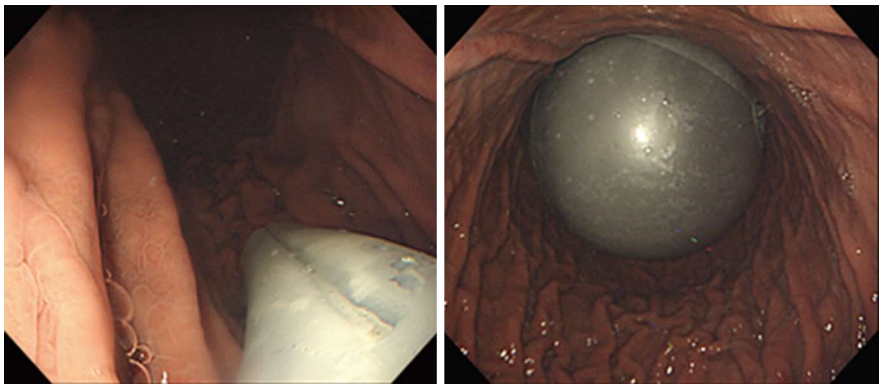

Figure 1 Intragastric balloon placement.

Table 1 Types of intragastric balloons

Balloon	Material	Volume	Weight loss[1]	Note
Fluid-supplied				
Orbera (Apollo Endosurgery)	Silicone	400-700 mL (saline)	16.9 ± 0.9 kg (at 6 mo)[58]	Most widely used and studied intragastric balloon Volume adjustment at the time of placement
Spatz Adjustable Balloon system (Spatz FGIA)	Silicone	400-600 mL (saline)	24 kg (at 12 mo)[64]	Totally adjustable balloon Approved for 12 mo of implantation
ReShape Duo® Integrated DualBalloon System (ReShape medical)	Silicone	900 mL (450 mL × 2) (saline)	25.1 ± 1.6 %EWL (at 6 mo)[62]	Two independent balloons connected to silicone shaft
The Elipse™ (Allurion Technologies)	N/A	450-550 mL (filling fluid)	2.4 kg (at 6 wk)[19]	Naturally swallowed, self-emptying, and naturally excreted
Air-supplied				
Obalon® Gastric Balloon (Obalon Therapeutics)	N/A	250 mL (air, nitrogen)	5 kg (at 12 wk)[66]	Can be swallowed A second or a third balloon can be swallowed depending on patient's progress
Heliosphere BAG® (Helioscopie)	Polyurethane and silicone	950 mL (air)	16 ± 7 kg (at 6 mo)[23]	Less than 30g

[1]Values extrapolated from representative reviews and clinical trials of each intervention. N/A: Not available; %EWL: Percentage of excess weight loss.

silicone balloon containing saline (450-700 mL) (Table 1). The positioning assembly, which comprises a balloon-filling tube and a catheter with the deflated balloon, is blindly advanced to the gastro-esophageal junction. An endoscopic device is inserted to ensure the precise deployment of the intragastric balloon, which is then filled with methylene-mixed saline under direct observation *via* the catheter. If an unexpected balloon rupture occurs, the methylene blue turns the urine green. The Orbera balloon is usually implanted for 6 mo, removed endoscopically by needle aspiration of the intragastric fluid, and retrieved with a snare or grasper. The FDA approved the use of the Orbera balloon on August 6, 2015. It is expected that the Orbera balloon could provide a valuable and less invasive therapeutic approach to bariatric treatment.

ReShape duo

The ReShape Duo® (ReShape Medical, San Clemente, CA, United States) aims to improve the space-occupying effects of intragastric balloons. This non-invasive device is delivered *via* the mouth through a 30-min endoscopic procedure. It is inflated with 900 mL of saline, which is equally distributed to each of two balloons. The balloon is inflated by a controller with methylene blue-mixed saline. This dual balloon system may reduce deflation-associated complications. If one of the balloons ruptures, the other balloon could sustain the location of the device in the stomach. It is recommended that the balloon be retrieved 6 mo after placement. The ReShape Duo was also approved by the FDA, on July 29, 2015.

Spatz

The Spatz Adjustable Balloon System (Spatz Medical, Great Neck, NY, United States) is a silicone balloon that is inflated with saline. It includes a filling catheter, which is extractable endoscopically, that permits an intragastric volume adjustment of 400-800 mL. The volume of the intragastric balloon can be modified to improve the patient's tolerance and increase weight reduction. The Spatz Adjustable Balloon System is allowed to be implanted in the stomach for 12 mo in locations outside the United States.

Elipse

The Elipse™ (Allurion Technologies, Wellesley, MA, United States) is a swallowable, self-draining, and naturally expelled intragastric balloon device for weight reduction. It is covered with a vegetarian shell and fixed to a flexible, slim tube. A resorbable substance inside the balloon degrades, allowing the balloon to empty naturally after a certain period. The deflated intragastric balloon is devised to be excreted *via* the digestive tract[19]. It is inflated with 550 mL of fluid and can be placed in the stomach for 4 mo. Endoscopic procedures are not needed for Elipse™ placement or removal.

Obalon

The Obalon Gastric Balloon® (Obalon Therapeutics Inc, Carlsbad, CA, United States) is a balloon filled with 250 mL of air. It has a self-sealing valve linked to a catheter and is set inside a gelatin capsule. The balloon is packaged in the capsule and swallowed, and the thin catheter is extended alongside the esophagus into the stomach. Fluoroscopy is used to define the location of the intragastric balloon, and the gelatin capsule disintegrates and releases the balloon. The balloon is then inflated using a gas-contained canister. The catheter is separated from the balloon and removed after balloon inflation.

Heliosphere bag

The Heliosphere Bag® (Helioscopie, Vienne, France) comprises a double-bagged polymer covered with an external silicone pouch. It is slowly filled with 960 mL of air for a final inflation volume of 700 mL[20,21]. The weight of the Heliosphere Bag is less than 30 g[22,23].

Silimed balloon

The Silimed Gastric Balloon is a spherical silicone balloon set inside a thin silicone sheath[24]. It is attached to the endoscope by a snare, advanced by an endoscope, and placed in the gastric fundus. It is filled with 650 mL of saline solution mixed with contrast dye and 10 mL of methylene blue.

Adjustable totally implantable intragastric prosthesis

The adjustable totally implantable intragastric prosthesis (ATIIP) is a polyurethane intragastric balloon that is rugby-shaped, 12 cm long, and has a volume of 300 mL when inflated with air[25]. This balloon is placed with an endoscopic percutaneous gastrostomy technique followed by the deployment of a subcutaneous totally implantable system through a surgical procedure. This method may reduce the likelihood of device dislocation and allows for balloon volume adjustment. The proximal balloon position in the gastric fundus-corpus lesion could affect gastric accommodation, neurohormonal process, and electrical action, thus modifying various control processes related to satiety[14].

INTRAGASTRIC BALLOON TREATMENT FOR OBESITY

Indications

The intragastric balloon may offer a minimally invasive and valuable method for managing obesity and related conditions. It is used to achieve weight loss in obese people, generally those with a body mass index (BMI) > 35 kg/m^2, or 30 kg/m^2 with certain comorbidities. Intragastric balloon treatment may play a different role in bariatric treatment based on the grade of obesity.

Preemptive therapy

Early intervention and preemptive therapy for weight reduction can be performed in obese patients (BMI $\geqslant$ 30 kg/m^2) at risk for disease development, at high risk for all-cause mortality, and with a high cardiovascular risk profile[26].

The objective of preemptive treatment is to achieve modest weight reduction, and, therefore, the overall risk/benefit ratio could validate the standards for procedures with this indication. Depending on the circumstances, indications for placement of intragastric balloons could also be extended to manage overweight people with a BMI < 30 kg/m^2 who desire weight reduction but who cannot achieve body weight loss with a controlled dietary program or with pharmacotherapy[27].

Metabolic therapy

Body weight loss achieved with intragastric balloon placement is associated with improvements in obesity-related metabolic illness. The intragastric balloon for metabolic therapy may be performed in patients with mild obesity (BMI $\geqslant$ 30 kg/m^2), where recovery from metabolic disease is the primary concern[28]. Co-existing illnesses, such as hyperlipidemia, type Ⅱ diabetes mellitus, and hypertension, could be particularly improved or resolved with even a modest reduction in body weight[29,30]. Treatment for metabolic issues should be relatively low-risk and have superior stability.

Primary therapy

The goal of intragastric balloon treatment is to achieve weight reduction in severely obese people, generally those with a BMI > 35 kg/m^2 with or without comorbidities, and who could not achieve long-term weight loss with a weight-control regimen. In addition, intragastric balloon therapy could be performed in patients with a BMI $\geqslant$ 40 kg/m^2, primarily as a preparation for bariatric treatment or in patients with increased surgical risks. Obese patients who reject bariatric surgical procedures or who do not have an approach for surgery can also opt for it. Intragastric balloon therapy used as a primary therapy could induce weight reduction and improve obesity-related comorbidities with a level of safety and efficiency

comparable to that of bariatric surgery[28]. However, lower efficiency is also acceptable because of the lower risk profile of intragastric balloon therapy.

Exclusion criteria

Exclusion criteria include any situation that could increase the risks related to intragastric balloon insertion, such as a large hiatal hernia (> 5 cm), active ulcer in the stomach or duodenum, previous surgical resection of the stomach, inflammatory bowel disease, gastrointestinal neoplasm, oropharyngeal abnormalities, active gastrointestinal bleeding, coagulative disorder, variceal disease, alcoholic disease or drug abuse, psychiatric disease, pregnancy, use of anti-coagulants or anti-inflammatory drugs, and cardiovascular, pulmonary or cerebrovascular diseases[31].

EFFECTS OF INTRAGASTRIC BALLOONS

The purpose of intragastric balloon treatment is to stimulate weight loss and assist with recovery from associated comorbidities with adequate safety. Gastric capacity restriction is an essential factor in surgical bariatric management.

Surgical gastric restriction could induce early satiety and potentiate gastric mechanical and chemical stimulation through a relationship with various gastric or exogenic factors. It affects hunger control and gastric emptying through alterations in gut hormones and peptides[32-34]. The intragastric balloon attempts to mimic surgical weight loss procedures by restricting the effective gastric volume.

Body weight loss

Results from previous studies indicated that the mean weight loss associated with intragastric balloon therapy ranged between 10.5 and 13.7 kg after 3 mo and between 12 and 26.3 kg after a 6-mo placement of the Orbera intragastric balloon[22,23,35-49]. After balloon removal at 6 mo, the achieved weight reduction endured to some extent. The excess weight loss (EWL) at the 12-mo implantation mark (6 mo after removal) was 14% to 50.9%[37,40,42,44,50-54]. The EWL at 12 mo after balloon extraction ranged from 14.2% to 27.2%[51,55-57]. The Orbera intragastric balloon was most effective during the first 3 mo of therapy. During that time, average weight loss of obese patients was 12.9 kg, or 80% of the total achieved weight reduction[58]. Additionally, the initial body weight loss (BWL) following intragastric balloon placement was associated with significant long-term weight maintenance. The percentage of BWL 1 mo after intragastric balloon placement was significantly associated with weight loss after 6, 12, and 18 mo (Pearson correlation coefficient = 0.77, 0.65, and 0.62, respectively, $P < 0.001$ for all)[59].

A study reported that insertion of a second balloon is not difficult and achieved good results. The mean %EWL was 31.5 ± 23.2 after the second balloon removal[43]. Some studies showed long-term results after intragastric balloon placement. A multi-center European study presented results for weight loss 3 years after balloon removal, which accounted for 29.1% of mean EWL[54]. A study that included 195 patients who were followed up for 5 years after intragastric balloon insertion demonstrated a %EWL of 12.97 ± 8.54[44].

Additional limited studies have utilized other intragastric balloons. A Spanish study with 60 patients showed a total body weight reduction of 16.6 ± 9.33 kg 6 mo after implantation of the ReShape Duo double-balloon system[60]. A prospective study with a double-balloon system showed a mean EWL of 18.3% in the control group compared to 31.8% in the treatment group 6 mo post-balloon implantation[61]. Furthermore, 64% of the reduced body weight was maintained at 12 mo post-implantation (6 mo after removal). In the REDUCE Pivotal Trial, a prospective randomized trial with 326 patients, patients assigned to the double-balloon system plus exercise and diet showed significantly superior EWL compared to the sham endoscopy plus exercise and diet alone group at 6 mo post-implantation (25.1% *vs* 11.3%, $P < 0.05$) in an intent-to-treat analysis[62].

A pilot trial showed 15.6 kg of mean weight loss at 24 wk and 24.4 kg at 52 wk after Spatz adjustable balloon placement[63]. A small observational investigation in 73 obese patients showed 45.7% of EWL 12 mo after deployment of the Spatz adjustable balloon[64].

Two preliminary studies with the Silimed Gastric Balloon reported results after the completion of 6 mo of treatment. The mean weight loss was 11.3 kg[24] and 8.1 kg[65]. A total of 57 morbidly obese patients underwent ATIIP placement. Mean EWL was 28.7% at 6 mo (38 patients) and 39.2% at 12 mo (20 patients)[25]. The Obalon (orally ingestable intragastric balloon) showed median weight losses after 4 weeks, 8 weeks, and 12 wk as 2.2 kg, 4.0 kg, and 5 kg, respectively[66]. A prospective study with 50 obese patients compared the effects of intragastric balloon therapy or pharmacotherapy on weight reduction. At 6 mo, patients in the intragastric balloon group had lost more weight than had patients in the pharmacotherapy group (percent of initial weight lost, %IWL = 14.5 ± 1.2; percent of excess BMI lost, %EBL = 37.7 ± 3.2 *vs* %IWL = 9.1 ± 1.5, %EBL = 25.3 ± 4.1, respectively, $P < 0.005$)[67].

Although the intragastric balloon has been shown to be effective in causing a meaningful weight loss, several studies have reported that the results were short-lasting, with most patients regaining weight following intragastric balloon removal[31,36,51,55,68-72].

Improvement in metabolic diseases

Obesity in patients is related to several comorbidities that are significant targets for obesity treatment. A

Table 2 Plasma ghrelin and leptin level after intragastric balloon treatment

Ref.	Balloon	Weight loss at 6 mo	Ghrelin (at 0 mo)	Ghrelin (after 3 mo)	Ghrelin (after 6 mo)	Ghrelin (after 12 mo)	Leptin (at 0 mo)	Leptin (after 3 mo)	Leptin (after 6 mo)	Leptin (after 12 mo)
Mathus-Vliegen *et al*[17]	Orbera	17.4 ± 7.8 kg	722.3 ± 151.5 pg/mL	791.5 ± 239.0 pg/mL	743.7 ± 115.2 pg/mL	N/A	N/A	N/A	N/A	N/A
Fuller *et al*[48]	N/A	14.2%	414.1 pmol/L	448 pmol/L	452.4 pmol/L	379.4 pmol/L	23.4 ng/mL	18.5 ng/mL	11.7 ng/mL	19.7 ng/mL
Bužga *et al*[85]	MedSil®	18.4 ± 8.2 kg	240.5 ± 101.5 μg/L	378.1 ± 155.8 μg/L	335.8 ± 149.2 μg/L	N/A	30.4 ± 17.2 μg/L	18.2 ± 15.8 μg/L	14.9 ± 15.5 μg/L	N/A
[1]Nikolic *et al*[86]	Orbera	N/A	958.3 pg/mL	1346.2 pg/mL	1050.1 pg/mL	922.6 pg/mL	25.1 ng/mL	14.3 ng/mL	10.5 ng/mL	17.5 ng/mL
Konopko-Zubrzycka *et al*[39]	Orbera	17.1 ± 8.0 kg	621.9 ± 182.4 pg/mL	903.9 ± 237 pg/mL (1 mo)	N/A	N/A	61.3 ± 36.7 ng/mL	39.9 ± 17.5 ng/mL (1 mo)	N/A	N/A
Martinez-Brocca *et al*[79]	Orbera	12.7 ± 5.6 kg (4 mo)	934.4 ± 199.2 pg/mL	947.1 ± 195.1 pg/mL (1 mo)	N/A	N/A	31.9 ± 16.4 ng/mL	22.4 ± 15.1 ng/mL (1 mo)	N/A	N/A
Mion *et al*[78]	Orbera	8.7 kg	3.2 ± 0.4 ng/mL	N/A	1.9 ± 0.1 ng/mL	N/A	27.8 ± 3.7 ng/mL	N/A	18.7 ± 2.7 ng/mL	N/A

[1]Data from patients with body mass indexes < 40 kg/m^2. Data are presented as mean ± SD or median. N/A: Not available.

study with 143 obese patients described the effects of the Orbera intragastric balloon at the 12-mo follow-up[51]. The incidence of metabolic syndrome declined from 34.8% (before balloon insertion) to 14.5%, 13%, and 11.6% at the time of removal, at the 6-mo follow-up, and at the 1-year follow-up, respectively. The incidence of type 2 diabetes mellitus decreased from 32.6% to 20.9%, 22.5%, and 21.3%, respectively. Likewise, the occurrence of hyperuricemia, hypertriglyceridemia, and hypercholesterolemia decreased from 26.1%, 37.7%, and 33.4% to 25.4%, 14.5%, and 16.7%, respectively, at the time of removal, 25.9%, 15.2%, and 16.7%, respectively, at the 6-mo follow-up, and 26.4%, 17.4%, and 18.9%, respectively, at the 1-year follow-up. A multi-center study presented data following treatment with the Orbera intragastric balloon in overweight populations[54]. The percentage of patients with co-morbidities at baseline and at the 3-year follow-up was 29% and 16% for hypertension, 15% and 10% for diabetes mellitus, 20% and 18% for dyslipidemia, 32% and 21% for hypercholesterolemia, and 25% and 13% for osteoarthropathy, respectively. A study with 119 obese patients assessed the effects of the Orbera intragastric balloon on obesity-associated diseases and quality of life[42]. Six months after placement of the intragastric balloon, the rate of metabolic syndrome in the patients decreased from 42.9% to 15.1% (P < 0.0005). Cholesterol, triglycerides, fasting glucose, C-reactive protein levels, and blood pressure also improved after balloon treatment (P < 0.005). In patients with diabetes mellitus, the HbA1c level was decreased at 6 mo compared to that at baseline (7.4% to 5.8%; P < 0.0005). In addition, the quality of life of patients increased.

Obesity is one of the risk factors for nonalcoholic fatty liver disease (NAFLD). About 27% of patients with NAFLD can develop fibrosis, and 19% can develop cirrhosis[73]. A randomized controlled study showed that intragastric balloon therapy improved the histology of nonalcoholic steatohepatitis[74]. Six months after implantation, a significant reduction in the median NAFLD activity scores was observed in the intragastric balloon-treated group (2 *vs* 4, P = 0.030) compared to that in the control group. Additionally, the median steatosis scores displayed a trend toward improvement in the balloon-treated group compared to those in the control group (1 *vs* 1, P = 0.075).

Alterations in gastrointestinal hormones

Body weight is controlled by multifaceted coordination of both central and peripheral factors. It is now obvious that a physiologic change in gut neurohumoral signaling is one important contributor to weight reduction and improvement in related diseases obtained by anatomic surgical manipulation of the gastrointestinal tract[75]. Moreover, intragastric balloon treatment may affect weight changes through an interaction of gastric neurohumoral factors (Table 2). These factors include several gut peptides and hormones, such as ghrelin, leptin, cholecystokinin, peptide YY, pancreatic polypeptide, and glucagon-like peptide-1.

These factors are interrelated and participate in the peripheral mediation of satiety[76,77]. Ghrelin is a hormone that has been known to influence energy balance. However, several studies presented varying results regarding ghrelin concentrations after balloon treatment. A study with 40 obese patients who underwent balloon placement indicated no effect on ghrelin levels when patients were fasting or meal-suppressed[17]. In another study, 17 patients with non-morbid obesity underwent balloon placement, and fasting plasma ghrelin concentrations significantly decreased (3.2 to 1.9 ng/mL; P = 0.021) as a result[78]. Martinez-Brocca *et al*[79] reported that fasting and meal-suppressed plasma ghrelin levels did not

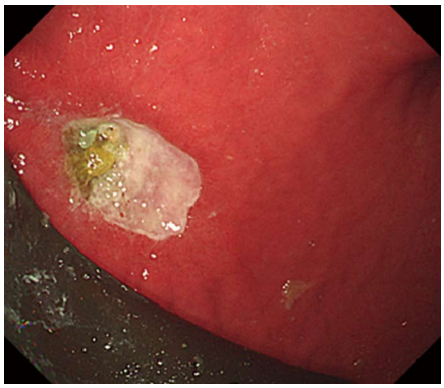

Figure 2 Gastric ulcer induced by intragastric balloon placement.

differ significantly between groups in morbidly obese patients. Konopko-Zubrzycka *et al*[39] reported that body weight reduction after balloon treatment is related to a transient elevation in plasma ghrelin levels and a decrease in plasma leptin levels.

Another study evaluated fasting and postprandial cholecystokinin and pancreatic polypeptide secretion after 13 wk of balloon treatment in obese patients. Baseline and meal-stimulated cholecystokinin levels were decreased[18].

Maintenance of weight loss is controlled by the collaboration of several processes, including environmental, behavioral, and homeostatic factors[80]. Physiological adaptation to weight reduction and weight regain show interplaying alterations in the stability of the levels of hunger hormones and energy homeostasis, in addition to changes in subjective appetite and nutrient metabolism. Appetite-related gastrointestinal hormones may play a crucial function in body weight regain after weight reduction[81]. Bariatric procedures may prove to be effective methods to aid in altering obese patients' physiology and may provide a good opportunity for long-term maintenance. Procedures that affect the physiology of weight loss and regain may eventually fortify future approaches for obesity treatment.

COMPLICATIONS OF THE INTRAGASTRIC BALLOON

Adverse events rates following Orbera intragastric balloon placement showed pooled incidences of pain (33.7%) and nausea (29%) as common adverse side effects[82]. The incidence of GERD, gastric ulcers (Figure 2), and balloon migration was 18.3%, 2%, and 1.4%, respectively. Serious adverse events with the Orbera balloon are uncommon, with prevalences of small bowel obstruction, perforation, and death as 0.3%, 0.1% and 0.08%, respectively[82]. Similarly, the REDUCE pivotal trial (n = 264), which evaluated the efficacy and safety of the ReShape Duo intragastric balloon, showed that symptoms such as nausea, abdominal pain, and vomiting were common, but decreased rapidly with medical management. Spontaneous intragastric balloon deflation occurred in 6% of patients, but balloon migrations did not occur concurrently. Early balloon removal occurred in 9.1% of study participants for non-ulcer-related intolerance. In 35% of the study participants, a gastric ulcer presented initially, but ulcer incidence and size decreased following subsequent device modification[62].

FUTURE DIRECTIONS FOR THE INTRAGASTRIC BALLOON

New emerging technology trends in endoscopic treatment for obesity require an extensive and meticulous research plan to promote finding and recognize their optimal role in managing obese patients and their applications for clinical practice[83]. Intragastric balloon placement can be performed through a simple endoscopic method and is easily reversible. This simplicity offers an expansive role in obesity treatment based on the degree of obesity. It is important to establish an appropriate method for intragastric balloon treatment by classifying the degree of obesity. Although serious complications with intragastric balloon treatment are rare, safety is an issue that cannot be overlooked. Various factors are associated with balloon safety, such as its durability and the simplicity of the procedure. Advances in device properties and procedural techniques could offer more cost-effectiveness with this outpatient procedure[84]. There is also uncertainty about the ideal filling medium and content. A preference for fluid other than air has been mentioned[14], but a debate still exists, with no established guidelines to date. There is a need to establish standards of intragastric balloon treatment practice, which could comprise pre- and post-procedural guidelines and long-term schedule management. In addition, collaboration with dietary counseling and an exercise program could offer quality assurance with intragastric balloon treatment.

Intragastric balloon treatment might produce only short-lasting effects in obesity treatment. Thus, it is important to maintain weight loss following intragastric balloon removal. Long-term management for weight reduction after intragastric balloon removal can also comprise intensive lifestyle modification, alone or with pharmacotherapy, and could be suggested to protect against weight regain.

Material and safety issues regarding the intragastric balloon should be further investigated. More research should be performed to investigate a pathophysiologic pattern of obesity, the uncertain role of gut hormones, potential predictive factors for the efficacy of the intragastric balloon in obesity treatment, and individualized treatment-induced changes.

CONCLUSION

Effective and safe weight reduction is very important

for the treatment of obesity, which is responsible for about 5% of all obesity-related deaths globally. Intragastric balloon treatment is more than just a transient curiosity. It shows promise in improving the quality of life and health status for obese patients. It offers a minimally invasive and effective method for managing obesity and associated conditions. Although there remains the possibility of improvement with research and development, gastroenterologists should maintain attention and interest in determining satisfactory outcomes and verifying standards of intragastric balloon treatment for the obesity epidemic.

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MINIREVIEWS

Role and mechanisms of action of *Escherichia coli* Nissle 1917 in the maintenance of remission in ulcerative colitis patients: An update

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Abstract

Ulcerative colitis (UC) is a chronic inflammatory disease, whose etiology is still unclear. Its pathogenesis involves an interaction between genetic factors, immune response and the "forgotten organ", Gut Microbiota. Several studies have been conducted to assess the role of antibiotics and probiotics as additional or alternative therapies for Ulcerative Colitis. *Escherichia coli* Nissle (EcN) is a nonpathogenic Gram-negative strain isolated in 1917 by Alfred Nissle and it is the active component of microbial drug Mutaflor® (Ardeypharm GmbH, Herdecke, Germany and EcN, Cadigroup, In Italy) used in many gastrointestinal disorder including diarrhea, uncomplicated diverticular disease and UC. It is the only probiotic recommended in ECCO guidelines as effective alternative to mesalazine in maintenance of remission in UC patients. In this review we propose an update on the role of EcN 1917 in maintenance of remission in UC patients, including data about efficacy and safety. Further studies may be helpful for this subject to further the full use of potential of EcN.

Key words: Ulcerative colitis; *Escherichia coli* Nissle; Metanalysis; Probiotic; Randomized trial; Inflammatory bowel disease

Core tip: *Escherichia coli* (*E. coli*) Nissle is a nonpathogenic Gram-negative strain used as a probiotic with very good quality paper assessing its bio-equivalence to mesalazine in maintaining remission in ulcerative colitis. Mechanisms of actions of this compound include immune-modulatory properties, reinforcement of intestinal barrier and inhibitory effect towards other pathogenic *E. coli*.

Scaldaferri F, Gerardi V, Mangiola F, Lopetuso LR, Pizzoferrato M, Petito V, Papa A, Stojanovic J, Poscia A, Cammarota G, Gasbarrini A. Role and mechanisms of action of *Escherichia coli* nissle 1917 in the maintenance of remission in ulcerative colitis patients: An update. *World J Gastroenterol* 2016; 22(24): 5505-5511 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i24/5505.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i24.5505

INTRODUCTION

Ulcerative colitis (UC) is a chronic remitting and relapsing disease, characterized by a continuous inflammation which can stretch from the rectum up to the entire colon, often resulting in mucosal ulceration, rectal bleeding, diarrhea, abdominal pain. Its etiology is still unclear, and it is multifactorial. Several factors have been identified as major determinants for induction or relapses and, among these, the imbalanced gut microbiota has become more crucial in recent years. The main hypothesis is that it is due to an excessive immune response to endogenous bacteria, in genetically predisposed individuals[1].

The human microflora, known as "microbiota", includes more then thousand different species and higher than 15000 different bacterial strains, for an average total weight of 1 kg. In recent years several studies investigated the correlation between dysbiosis and intestinal and extra-intestinal diseases, including inflammatory bowel disease (IBD) and so UC[2].

Probiotics are viable agents conferring benefits to the health of the human host[3]. They can provide a beneficial effect on intestinal epithelial cells in numerous ways. Some strains can block pathogen entry into the epithelial cell by providing a physical barrier or by creating a mucus barrier; other probiotics maintain intestinal permeability acting on tight junctions. Some probiotic strains produce antimicrobial factors, other strains modulate the immune response[4].

The role of microbiota in UC was supported by several evidences: inflammation is greatest in intestinal tracts with high concentration of bacteria, surgical reduction of the bacterial load is associated with improvement of inflammation and inflammation does not occur in germ free animals[5].

The treatment goal of UC is the induction and the maintenance of remission. 5-aminosalicylic acid (ASA) compounds, azathioprine/6 mercaptopurine, corticosteroids, cyclosporine, methotrexate, and anti-TNFα agents are conventional therapies used to control the disease. There are also other therapies, used as addition or alternative to conventional therapies in UC, in particular antibiotics and probiotics, which modulate gut microbiota.

Escherichia coli (*E. coli*) Nissle (EcN) 1917 is a non-pathogenic Gram-negative strain used in many gastrointestinal disorder including diarrhea[6], uncomplicated diverticular disease[7] and IBD, in particular UC[8].

STRUCTURE AND MECHANISMS OF ACTION OF EcN 1917

EcN 1917 (O6:K5:H1) was isolated by Prof. Alfred Nissle from Freiburg, Germany, in 1917 from the intestinal microflora of a young soldier. This soldier - unlike his comrades - did not develop infectious diarrhea, when stationed during World War I in Southeastern Europe (Dobrudja/Balkan peninsula), endemic for Shigella at that time. The strain was named *E. coli* strain Nissle 1917.

Using this *E. coli* strain Prof. Nissle developed the probiotic drug Mutaflor® and introduced it into medical practice in the same year. Since 1917, Mutaflor® is available in the German pharmaceutical market without interruption and recently also in Italy as EcN (cadigroup).

The lack of defined virulence factors (alpha-hemolysin, P-fimbrial adhesins, *etc.*) combined with the expression of fitness factors such as microcins, different iron uptake systems (enterobactin, yersiniabactin, aerobactin, salmochelin, ferric dicitrate transport system, and the *chu* heme transport locus), adhesins, and proteases may support its survival and efficacious colonization of the human gut, and contribute to the probiotic character of EcN 1917.

It exhibits a semi-rough lipopolysaccharide (LPS) phenotype and serum sensitivity and does not produce known toxins[9]. EcN colonizes the intestine within few days and it remains as colonic flora for months after administration[10].

EcN has an intestinal anti-inflammatory effect, but also systemic effects[11], and there are many theories about its mechanism of action (Figure 1): (1) It has direct antimicrobial effects: it inhibits EHEC (*E. coli* EDL933) colonization in animal models[12] and synthesis of Shiga-Toxins in co-cultivation experiment with STEC (Shiga-Toxin producing *E. coli*)[13]. (2) It is involved in the bacterial-epithelial crosstalk ("Host cell signaling") by biofilm formation: it expresses F1C Fimbria. This is very important in the formation of biofilm, adherence to epithelial cells and persistence in infant mouse colonization[14]. Its flagellum is the major "propulsor" *in vivo*, which allow this probiotic strain to efficiently compete with pathogens for binding sites on host tissue[15]. It directly stimulates

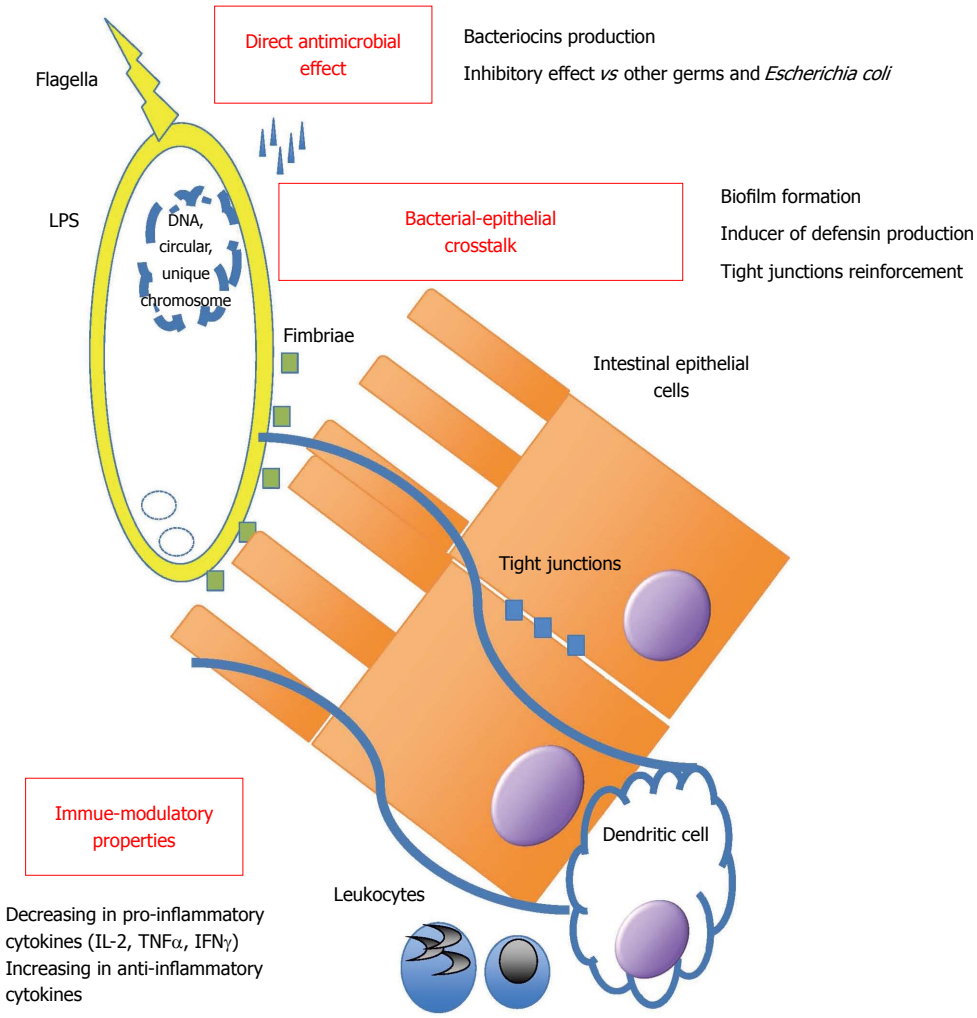

Figure 1 Structure and mechanisms of action of *Escherichia coli* Nissle 1917. LPS: Lipopolysaccharide; IL-2: Interleukin-2; TNF: Tumor necrosis factor; IFN: Interferon.

defensin production by intestinal epithelial cells, such as the human beta-defensin that inhibits adhesion and invasion of intestinal cells by pathogenic adherent invasive *E. coli*, which play a key role as trigger in immune response in IBD patients[16-18]. It strengthens tight junctions of intestinal epithelial cells, by up-regulating the expression of the mRNA for the zonula occludens proteins ZO-1 and ZO-2, so it has an effect on the repair of the "leaky gut"[19-21]. (3) It interacts with immune system by causing decreasing in pro-inflammatory cytokines (IL-2, TNF-α, IFNγ) and increasing in anti-inflammatory cytokines by peripheral blood mononuclear cells *in vitro*. It may reduce the expansion of newly recruited T cells into the intestinal mucosa and decrease intestinal inflammation, but it doesn't affect activated tissue-bound T cells, which may eliminate deleterious antigens in order to maintain immunological homeostasis[22,23]. Furthermore EcN has a specific LPS that is responsible for its immunogenicity, without major immunotoxic properties at doses suggested and that provide, together with the other described features, a powerful effect on intestinal immune function[24].

Efficacy of EcN in animal models of colitis

Schultz *et al*[25] conducted a study on animal models of acute and chronic colitis. Acute colitis was induced by administration of dextran-sodium sulfate (DSS) in drinking water and chronic colitis was induced by transferin $CD4^+$ $CD62L^+$ T lymphocytes from BALB/c mice in SCID mice. These studies have shown that administration of EcN ameliorates intestinal inflammation (measured by histological scores) in chronic models but not in acute models, in accordance with clinical observations. Therefore, it was shown that EcN reduced secretion of pro-inflammatory cytokines, measured by enzyme-linked immune-sorbent assay[25].

Grabig *et al*[5] demonstrated that EcN ameliorates experimental colitis induced by administration of 5% DSS in mice *via* TLR-2 and TLR-4 dependent pathway.

Decreasing of symptom scores and differences in body mass loss were shown in animal models of DSS colitis in BALB/c mice treated with EcN in the study conducted by Kokesová *et al*[26].

Table 1 Main results from trials on *Escherichia coli* Nissle on ulcerative colitis

Efficacy of EcN 1917 in maintanance of UC remission
Results from major randomized controlled clinical trials
EcN 200 mg/d is equivalent to Mesalazine 1000 mg/d in mantainance of UC remission[27]
EcN 400 mg/d is equivalent to to Mesalazine 2400 mg/d in maintanance of UC remission following an acute flare[28]
EcN 200 mg/d is equivalent to Mesalazine 1500 mg/d in mantainance of UC remission[10]
Results from minor studies
Rectal admistration of EcN 40 mL/d is effective in moderate distal active UC[30]
EcN 200 mg/d is equivalent to Mesalazine 1500 mg/d in maintanance of UC remission[6]

EcN: *Escherichia coli* Nissle; UC: Ulcerative colitis.

CLINICAL ROLE OF EcN 1917 IN MAINTANANCE OF UC

There are three major double-blind RCTs (Table 1), which compare EcN to mesalazine in prevention of relapse in UC patients, all of them designed to demonstrate equivalence of two treatment according to Schuirmann's two-one side test or "non inferiority trials".

The first trial was conducted by Kruis *et al*[27] in 1997. It was a randomized, double-blind, double-dummy study conducted on 120 out-patients in Germany, Czech Republic and Austria. Patients had a confirmed diagnosis of ulcerative colitis in remission. In particular patients had to be in remission for a maximum of 12 mo, with clinical activity index (CAI) < 4, no endoscopic or histological signs of acute inflammation. Each patient had to have had at least 2 relapses prior inclusion.

Patients received 500 mg mesalazine t.d.s. and a placebo form of EcN preparation or 200 mg/d of a preparation containing EcN in a single dose ("Mutaflor", Ardeypharm GmbH, Herdecke, Germany. 100 mg contains 25×10^9 viable *E. coli* bacteria) and a placebo form of mesalazine. The duration of the study was 12 wk. Study objectives included the assessment of the equivalence of the CAI under the two treatment modalities and the comparison of the relapse rates, relapse-free times and global assessment.

Study population was homogeneous into the two groups, with a prevalence in left sided colitis and small prevalence of active use of steroids (less then 25 % in both groups).

From the results of this study, no significant difference was observed between the two groups, although a low statistical power and a minor trend towards a slightly higher CAI in the EcN group. No serious adverse events reported for both groups.

The second trial was conducted by Rembacken *et al*[28] in 1999. This was a single-center, randomised, double-dummy study involving 116 patients, which were treated with mesalazine 800 tds (Asacol formulation) or EcN 2 cp per 2 times daily ("Mutaflor", Ardeypharm GmbH, Herdecke, Germany. 100 mg contains 25×10^9 viable *E. coli* bacteria).

Inclusion criteria were: 18-80 years of age, clinical active (mild-moderate and severe) ulcerative colitis ("Leeds-Index") defined by number of 4 or more liquid stools/day for the last 7 d, with or without blood, erythema on sigmoidoscopy and histological confirmation of active ulcerative colitis.

The study populations were comparable. In particular: the median clinical activity index on study entry was 11 in the mesalazine group and nine in the EcN group (up to 30% of patients had a severe disease). The median sigmoidoscopy score was four in both groups. All patients received rectal or oral steroids at different doses together with a 1-wk course of oral gentamicin. At baseline both groups had an high active usage of steroids, around for 50% of the cases; furthermore, 2% in mesalazine group and 18% in EcN. In both groups the proportion between proctitis, left sided and pancolitis was similar (1/3 per each condition). No significant differences between the 2 groups were found.

Active treatment, started at the enrolment, was hydrocortisone enema twice daily, prednisolone 30 mg a day in moderate colitis and prednisolone 60 mg a day in severe colitis (according to Truelov Witts criteria). Only people in remission at 12 wk were enrolled in the follow up part of the trial assessing maintenance of remission.

Starting from remission, patients were maintained on either mesalazine or *E. coli* but the doses were reduced respectively at 1.2 g per day for the mesalazine group and 2 capsules per day for the EcN group. The follow up was at 12 mo. 59 were randomised to mesalazine and 57 to EcN. Of them 75% and 68% reached remission. Of those in remission, 73% of patients in the mesalazine group and 67% in the EcN group, relapsed by 12 mo. In the mesalazine group, the mean duration of remission was 206 d (median 175) compared with 221 (median 185) in the group given *E. coli*, ($P = 0.0174$). The treatment with EcN was proved safe, acceptable, and clinically equivalent to mesalazine in maintaining remission after an acute relapse of ulcerative colitis. Finally, this study is characterized by a very high rate of relapse: this is however not un-expected as the study population comprehends moderate and also severe patients.

The third trial was conducted by Kruis *et al*[10] in 2004. This was a double-blind, double dummy study in which 327 patients affected by ulcerative colitis in remission phase were recruited. 162 patients received Mutaflor 200 mg/d and 165 received Mesalazine 500 mg three times daily for 12 mo. Inclusion criteria were: age between 18-70 years, diagnosis of UC in remission [CAI $\leqslant$ 4, endoscopic index (EI) $\leqslant$ 4, and no signs of acute inflammation on histological examination]. Furthermore, within inclusion criteria there was at

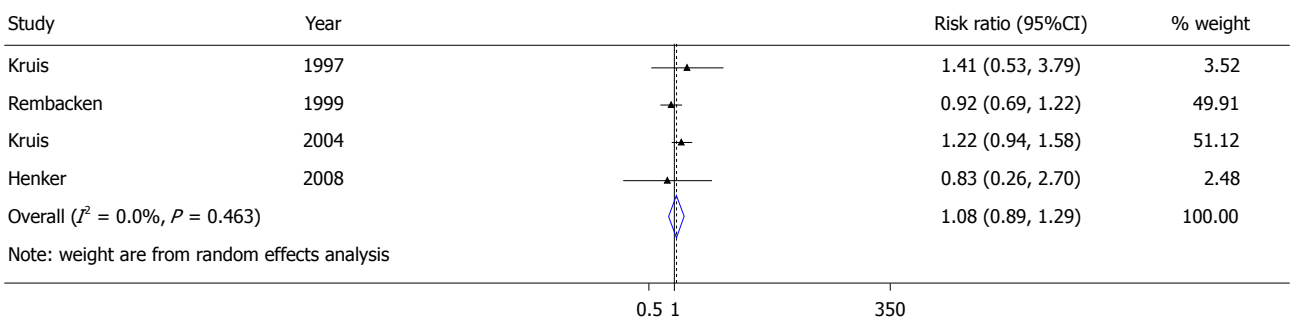

Figure 2 Metanalysis on randomized controlled trials assessing role of *Escherichia coli* Nissle on maintenance of remission in ulcerative colitis.

least two acute attacks of UC prior to the study and duration of the current remission of no longer than 12 mo. Primary objective of the study was meant to compare the number of patients experiencing a relapse during the 12 mo observation time between the two treatment groups. Secondary aims included efficacy variables like physician's and patient's assessment of general well being and calculation of a quality of life index. Additionally, time to relapse, CAI, EI, and histological findings were also evaluated. In the EcN group 36.4% of patients relapsed compared to 33.9% in the mesalazine group and statistical tests showed equivalence of the two treatments. A subgroup analyses showed no difference in terms of duration and localization of disease or pre-trial treatment. No difference of quality of life was shown in the two groups.

Overall same results on tolerance were found: it was very good or good in the EcN group in 80.0% and in the mesalazine group in 86.0%. According to the physician's assessment, the respective values were 85.1% and 90.3%. No unexpected drug reactions occurred during the study.

This is perhaps the best study based on the quality of data and also the large number of patients enrolled. Furthermore clinical outcomes were assessed by well-established endoscopic and histological activity indices, like in modern trials for more powerful drugs.

In addition to the above-described trials, there is a multicentric placebo-controlled study on 90 patients with moderate distal active UC conducted by Matthes *et al*[29] Patients in EcN groups received EcN 40, 20 or 10 mL (amount of bacteria 10E8/mL) enema once daily for at least 2 wk. A clinical DAI was recorded after 2, 4 and/or 8 wk.

The majority of patients also received concomitant medical treatment such as oral mesalazine. Remission rates and improvement of the histological score was showed particularly in the EcN 40 mL group, but further studies on largest population are required.

This study has shown that rectal administration of EcN is an effective treatment, with a dose dependent efficacy as shown in the Per Protocol analysis. Unfortunately the ITT analysis did not show significant results[29].

Many meta-analysis present in the literature support the role of ECN in the therapy of ulcerative colitis[6,10,27,28,30]. In particular, a very recent published meta-analysis, performed by Losurdo *et al*[31] (Figure 2), showed a non-significant inferiority of EcN in relapse prevention compared to mesalazine in preventing disease relapse, thus confirming current guideline recommendations[31], despite a novel randomized double-blinded placebo controlled trial conducted in Denmark and published very recently[32], with negative results. One hundred patients with active UC defined by CAI-score $\geqslant$ 6 and with calprotectin higher than 50 mg/kg, were enrolled and randomized into four groups of treatment: Ciprofloxacin (for 1 wk) followed by EcN (for 7 wk), Ciprofloxacin (for 7 wk) followed by placebo (for one week), placebo (for one week) followed by EcN (for 7 wk) and placebo (for one week) followed by placebo (for 7 wk). Aim of the study was the induction of the remission in ulcerative colitis and Kaplan-Meier curves were used to compare groups. In this study, the 54% of patients in the placebo/EcN group reached remission, compared to 89% of patients in the placebo/placebo group (P < 0.05), 78% of Ciprofloxacin/placebo group and 66% Ciprofloxacin/EcN group. Furthermore, the placebo/EcN group had the largest number of withdrawals. These impressive results, which would exclude a role of EcN in treatment of active ulcerative colitis, display several limitations, which make this study really weak. First of all this is a monocenter study, with a very not homogeneous population as showed in the table of patients characteristics. Mean CAI score at baseline was 10.5, 8.9, 9.3 and 8.9 in the Cipro/EcN, Cipro/placebo group, placebo/EcN group, placebo/placebo group, respectively. Furthermore patients clearly differed in concomitant medications use, in particular for use of active use of topical drugs and steroids as well as immunosuppressant. Taken together, these data suggest that this trial display major limitations regarding groups homogeneity. We confirmed the data from the published metanalysis, which we performed independently before discovering that it was just published. In the present paper we report an extract of the recent published metanalysis on the equivalence of the treatment between ECN and mesalazine[31],

Table 2 Main potential clinical indications for *Escherichia coli* Nissle in gastroenterology

Maintenance of remission in ulcerative colitis[6,10,27,28]
Irritable bowel syndrome[34-37]
Constipation[38,39]
Acute diarrhea[6,40]
Collagenous colitis[41]
Uncomplicated diverticular disease[7]

starting from the major trials available. An equivalence between EcN and mesalamine on maintenance of remission in UC is still detectable (Figure 2).

Other studies assessing the use of EcN in ulcerative colitis

There is also an open-label multicenter pilot study that investigate the clinical benefit of EcN 1917 for maintenance therapy in young patients with UC. In this study 34 patients with UC in remission aged between 11 and 18 years were allocated either to EcN (2 capsules daily n = 24) or 5-ASA (median 1.5 g/d, n = 10), and observed over one year[33]. Inclusion criteria were: 11-18 years of age, ulcerative colitis in remission for a maximum of 12 mo, at least 2 relapses prior inclusion, active therapy with mesalazine. Taking into account the low statistical power of the study, relapse rate was 25% (6/24) in the EcN group and 30% (3/10) in the 5-ASA group. Data on the patients' global health and development were favorable and no serious adverse events were reported[33].

CONCLUSION

EcN is a well known probiotic, used in several countries for GI diseases (Table 2)[6,10,27,28,34-41], registered as a drug in certain European countries, and it is the only one approved for maintenance of remission in UC patients by ECCO guidelines, based on data discussed also in the present paper. Trials designed to be non-inferiority/equivalence trials, comparing EcN to mesalazine, have reported equivalent rates of relapse between the two treatments, demonstrating that EcN is equivalent to mesalazine in the maintenance of remission in UC. Of the 3 major trials demonstrating these findings, the best and larger trial is the one conducted by Kruis *et al* and published on 2004. Finally, EcN showed a robust safe profile in UC patients. Further studies may be helpful to further dissect mechanisms of actions and perhaps optimize dose and newer indication of EcN.

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ORIGINAL ARTICLE

Basic Study

Transient receptor potential vanilloid 4-dependent calcium influx and ATP release in mouse and rat gastric epithelia

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Abstract

AIM: To explore the expression of transient receptor potential vanilloid 4 (TRPV4) and its physiological meaning in mouse and rat gastric epithelia.

METHODS: RT-PCR and immunochemistry were used to detect TRPV4 mRNA and protein expression in mouse stomach and a rat normal gastric epithelial cell line (RGE1-01), while Ca^{2+}-imaging and electrophysiology were used to evaluate TRPV4 channel activity. ATP release was measured by a luciferin-luciferase assay. Gastric emptying was also compared between WT and TRPV4 knockout mice.

RESULTS: TRPV4 mRNA and protein were detected in mouse tissues and RGE1-01 cells. A TRPV4-specific agonist (GSK1016790A) increased intracellular Ca^{2+} concentrations and/or evoked TRPV4-like current activities in WT mouse gastric epithelial cells and

RGE1-01 cells, but not TRPV4KO cells. GSK1016790A or mechanical stimuli induced ATP release from RGE1-01 cells while TRPV4 knockout mice displayed delayed gastric emptying *in vivo*.

CONCLUSION: TRPV4 is expressed in mouse and rat gastric epithelium and contributes to ATP release and gastric emptying.

Key words: Transient receptor potential vanilloid 4; Stomach; Gastric emptying; ATP

Core tip: A mechano-sensitive ion channel, transient receptor potential vanilloid 4 (TRPV4), is expressed in gastric epithelium and contributes to ATP release and gastric emptying. These findings suggest that gastric distension stimulates TRPV4 on gastric epithelium and released ATP stimulates sub-epithelial nerve fibers or acts on visceral smooth muscles. TRPV4 might be a promising novel diagnostic and therapeutic target for functional gastric disorders.

Mihara H, Suzuki N, Boudaka AA, Muhammad JS, Tominaga M, Tabuchi Y, Sugiyama T. Transient receptor potential vanilloid 4-dependent calcium influx and ATP release in mouse and rat gastric epithelia. *World J Gastroenterol* 2016; 22(24): 5512-5519 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i24/5512.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i24.5512

INTRODUCTION

The transient receptor potential vanilloid 4 channel (TRPV4) is a non-selective cation channel that is involved in various cellular functions[1] and is activated by several physical and chemical stimuli, including mechanical stimuli, endogenous arachidonic acid metabolites (epoxyeicosatrienoic acids)[2], and heat. TRPV4 is also activated by the specific agonist GSK1016790A that elicits whole-cell currents in mouse and rat TRPV4-expressing cells with EC_{50} values of 18.5 and 10 nmol/L, respectively[3]. TRPV4 is widely expressed throughout the body, including gastrointestinal tract epithelium and the esophagus[4]. Although the physiological function of TRPV4 expression in intestinal epithelial cells is unknown, TRPV4 activation in these cells causes increases in intracellular calcium concentrations, chemokine release, and incidence of colitis[5], as well as increased paracellular permeability[6]. Furthermore, TRPV4 antagonists are promising therapeutic options for colitis[7,8]. However, TRPV4 expression in the gastric epithelium awaits evaluation.

In addition to its function as an intracellular energy donor, ATP is recognized as an important signaling molecule that mediates diverse biological effects *via* cell surface receptors: the purinergic receptors[9]. ATP is released by neurons of the central, peripheral, and enteric nervous system[10,11], and acts as a non-adrenergic non-cholinergic (NANC) neurotransmitter that causes different responses or effects (either excitatory or inhibitory depending on the P2 receptor subtype upon which they act as well as the animal species under study). Several studies showed that purinergic neurotransmission (assuming that gut neurons are the sole source of released ATP) affects gastric motility[12]. Recent reports showed that ATP is also released from non-neuronal tissues and has an effect on tissue function. Moreover, we found that ATP release in the esophagus and urothelium was mediated by TRPV4 stimulation[4,13,14]. However, there are no data concerning whether TRPV4 is expressed in the stomach and, if so, whether TRPV4 stimulation plays a role in mediating ATP release. Therefore, this study explored the morphological (RT-PCR and immunostaining) and functional (Ca^{2+}-imaging, patch clamp and gastric emptying) expression of TRPV4 in mouse and rat stomach with special focus on gastric epithelium.

MATERIALS AND METHODS

Animals

Eight week-old male C57BL/6NCr (SLC) and TRPV4-knockout (TRPV4KO) mice[15] weighing between 23 and 25 g were housed in a controlled environment (12-h light/12-h dark cycle; room temperature, 22-24 ℃; 50%-60% relative humidity) with free access to food and water. All procedures involving the care and use of animals were approved by The Institutional Animal Care and Use Committee of the National Institutes of Natural Sciences.

Cell lines

RGE1-01 is an immortalized rat gastric mucosal cell line that shows distinct cell differentiation types and preserves some epithelial cell characteristics. RGE1-01 cells were maintained at 34 ℃ in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/mL streptomycin and 100 U/mL penicillin with the addition of ITES (see reference[16] for details).

Acute isolated mouse gastric epithelium

WT and TRPV4KO mice were sacrificed by cervical dislocation. The stomachs were washed in cold (4 ℃) PBS (-) and then incubated in trypsin solution (Invitrogen) at 4 ℃ for 1 h. Gastric epithelial cells were harvested and plated on CELL-TAK (BD Biosciences)-coated glass cover slips and used for Ca^{2+}-imaging and patch clamp experiments.

Reverse transcription PCR analysis

RT-PCR was performed as previously described[4,17]. Total RNA (1 μg) was isolated using the RNeasy Mini

Table 1 Primer sequences for RT-PCR

Primer name	Sequence (5'→3')
mTRPV4-F	ACAACACCCGAGAGAACACC
mTRPV4-R	CCCAAACTTACGCCACTTGT
mGAPDH-F	TGAAGGGTGGAGCCAAAAGG
mGAPDH-R	GGAAGAGTGGGAGTTGCTGTTG
rTRPV4-F	CCTGGCAGGGATCGAGGCCT
rTRPV4-R	GGATGGTGGTGGCCCACTGC
rGAPDH-F	GCCAAGGCTGTGGGCAAGGT
rGAPDH-R	GAGCAATGCCAGCCCCAGCA

Kit (Qiagen, Courtaboeuf, France) and measured with a NanoDrop device (Thermo Fisher Scientific Inc., Wilmington, United States). Genomic DNA was eliminated in the process of reverse transcription (QuantiTect Reverse Transcription Kit, QIAGEN). PCR was performed using rTaq DNA polymerase (TaKaRa) in an iCycler (Bio-Rad) with specific primer sets (Table 1).

Immunochemistry

Immunochemistry was performed as previously described[4] using the antibodies summarized in Table 2. For section preparation, mouse stomachs were fixed at 4 ℃ for 6 h. Tissues were placed in PBS-sucrose and embedded in OCT compound (Tissue Tek, Elkhart, IN, United States). Non-specific antibody binding was reduced by incubation in BlockAce (Yukijirushi, Sapporo, Japan) for 1 h at room temperature prior to antibody exposure. Preparations were analyzed using a confocal laser scanning microscope (LSM 700, Carl Zeiss). For immunocytochemistry, RGE1-01 cells were fixed at 4 ℃ for 20 min with the same fixative. Bovine serum albumin (3% BSA; Sigma) was used as a blocking solution.

Ca^{2+}-imaging

Fura-2 fluorescence was measured in primary mouse gastric epithelial cells and RGE1-01 cells with a standard bath solution containing 140 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L $MgCl_2$, 2 mmol/L $CaCl_2$, 10 mmol/L HEPES, and 10 mmol/L glucose at pH 7.4 (adjusted with NaOH) at 25 ℃. Results are presented as ratios of fluorescence intensities obtained with fura-2 emissions at 340 nm and 380 nm. GSK1016790A[3] and ionomycin (both from Sigma) were used as a TRPV4 agonist and a positive control, respectively. F_{340}/F_{380} was calculated and acquired with an image processing system (IP-Lab, Scanalytics Inc., Rockville, MD or AQUA COSMOS, Hamamatsu Co., Japan) and ImageJ software (http://rsb.info.nih.gov/ij/). Changes in ratio (Δ) were calculated by subtracting the mean basal values from peak values. Since the degree of responses (strong, weak, or no response) to GSK varied from cell to cell in WT gastric epithelial cells, we expressed the observed changes in all ionomycin-responsive cells as a ratio between WT and TRPV4 knockout mice (Δ). We evaluated 53 and 41 ionomycin-responsive cells from six WT mice and five TRPV4 knockout mice, respectively. Given the variations in response times, and that some WT cells responded 30 s after GSK application, we decided to incubate cells with GSK for 90 s.

Table 2 Primary and secondary antisera for immunochemistry

Tissue antigen/host	Dilution	Source
TRPV4/rabbit	1:500	B. Nilius, or Abcam
Goat anti-rabbit IgG-Alexa488	1:1500	Invitrogen, Inc.

Electrophysiology

The standard bath solution was the same as that used in the Ca^{2+}-imaging experiments. Pipette solutions for whole-cell recordings contained 140 mmol/L KCl, 5 mmol/L EGTA and 10 mmol/L HEPES, pH 7.4. Whole-cell recording data on primary gastric epithelial cells three hours after insolation and RGE1-01 were sampled at 10 kHz and filtered at 5 kHz for analysis (Axon 200B amplifier with pCLAMP software, Molecular Devices, Foster City, CA, United States). Voltage ramp-pulses from -100 mV to +100 mV (500 ms) were applied every 5 s to generate an I-V curve.

ATP release measurement

ATP concentrations released from RGE1-01 rat gastric epithelial cells cultured in 12-well plates or stretch silicon chambers (STB-10-04 from STREX Inc., Osaka, Japan) were measured by a luciferin-luciferase assay (ATP Bioluminescence assay kit CLS II, Roche Diagnostics) and a luminometer (Lumat LB 9507, Berthold Technologies, Japan), using a previously described method that was slightly modified[4]. For chemical stimuli, cells cultured to 70%-80% confluence and incubated in 500 μL bath solution for 30 min at room temperature (25 ℃) were used to measure basal ATP release. The superfusate was collected and replaced gently with another 500 μL of bath solution with or without the TRPV4 agonists GSK1016790A or 5,6-EET. The superfusate was collected after 15 min and the ratio of released ATP (15 min stimulation/30 min basal condition) was calculated. An aliquot (200 μL) of superfusate was then mixed with 200 μL luciferin-luciferase reagent for luminometric ATP measurements. For mechanical stimuli, stretching was quantitatively applied with a STB-10 stretch machine (STREX Inc.) to RGE1-01 cells cultured on a silicon chamber. Three minutes after chamber placement in the stretch machine, the superfusate was washed away to exclude artificial ATP release and replaced with 500 μL of bath solution for basal ATP measurement. After a further 3 min, the superfusate was collected and replaced, whereupon mechanical stimuli was applied for 3 min and the ratio of released ATP (3 min stimulation/3 min basal condition) was calculated. To block TRPV4 channels, cells were pre-treated with the specific TRPV4 antagonist HC 067047 (Sigma, 1 μmol/L) for 3 min[18].

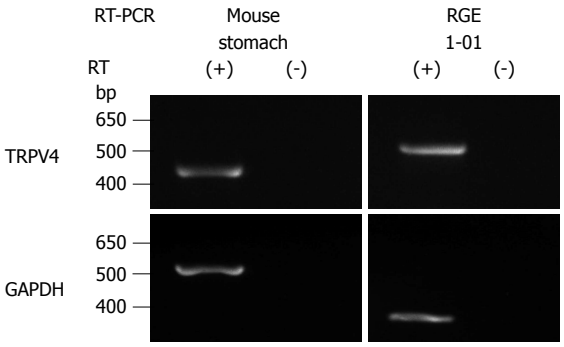

Figure 1 *TRPV4* mRNA expression in mouse stomach and RGE1-01 cells. *TRPV4* and *GAPDH* mRNA levels were examined with (+) and without (-) RT reaction. The expected sizes of the amplified fragments for *TRPV4* and *GAPDH* were 404 and 545 bp for mouse and 524 and 268 bp for rat, respectively. *TRPV4* mRNA was detected in mouse stomach and RGE1-01 cells (RGE1-01: normal rat gastric epithelial cell line). Band positions differed due to the use of different primers.

Gastric emptying

Eight-week-old WT and TRPV4KO male mice were used with a modified version of previously reported methods[17]. Gastric emptying was determined by transit of a test meal containing phenol red. Mice were fasted for 14 h with *ad libitum* water before the experiment. Five mg/kg (200 μL) of the test meal was administered into the stomach using a feeding needle. Fifteen minutes later, the mice were euthanized by cervical dislocation and the gastrointestinal tract was removed. The stomach was minced and the remaining phenol red concentration was measured. Gastric emptying was expressed as mean ± SEM for each group.

Data analysis

Values for Ca^{2+}-imaging, patch-clamp experiments, ATP measurements, and gastric emptying are presented as mean ± SEM from three or more independent experiments. A Student's *t*-test or non-parametric Bonferroni-type multiple comparison was used. Significance was accepted for $P < 0.05$.

RESULTS

TRPV4 expression in mouse and rat gastric epithelia

Given that TRPV4 was shown to be expressed in the esophagus and intestinal epithelia[4-6,19,20], we examined *TRPV4* mRNA expression in mouse stomach and a rat gastric epithelial cell line, RGE1-01. *TRPV4* mRNA was detected in mouse stomach and RGE1-01 cells (Figure 1). We next examined TRPV4 protein expression in mouse and the RGE1-01 cells. A strong homogenous immunofluorescent signal was confined to the epithelial cell layer of the WT mouse gastric corpus and antrum but not in cells from TRPV4KO mice (Figure 2A). Meanwhile, Z-stack images obtained by confocal microscopy of RGE1-01 cells displayed apical TRPV4 expression (Figure 2B).

TRPV4-mediated increase in cytosolic Ca^{2+} ($[Ca^{2+}]_i$) in mouse primary gastric epithelial cells

To confirm functional TRPV4 expression in primary gastric epithelial cells and RGE1-01 cells, we examined the response to the reported specific TRPV4 agonist, GSK1016790A (GSK)[3], using a fluorescent Ca^{2+}-imaging system (10 μmol/L, fura-2/AM). Response traces of $[Ca^{2+}]_i$ for WT and TRPV4KO gastric epithelial cells in the presence of GSK (100 nmol/L) showed that almost all cells isolated from WT stomach responded to GSK (Figure 3A) and the $[Ca^{2+}]_i$ increases were significantly larger in WT cells compared to TRPV4KO cells (Figure 3B). This finding suggests that the majority of gastric epithelial cells expressed TRPV4 and $[Ca^{2+}]_i$ responses to GSK were TRPV4 specific.

TRPV4-mediated current responses in mouse primary gastric epithelial cells and RGE1-01 cells

We next performed patch-clamp experiments with acute isolated mouse gastric epithelial cells in the presence of GSK (300 nmol/L) and observed inward current responses with an outwardly rectifying IV-relationship in WT but not TRPV4KO cells (Figure 4A)[3]. Current responses were observed in all 5 trials with WT gastric epithelial cells but were completely absent with TRPV4KO cells, which indicates that the majority of gastric epithelial cells expressed TRPV4. Similar chemical stimulation with GSK (300 nmol/L) induced TRPV4-like current responses in the rat gastric epithelial cell line, RGE1-01 (Figure 4B). These data strongly indicated functional expression of TRPV4 in mouse and rat gastric epithelial cells.

TRPV4 activators induced ATP release from RGE1-01 cells

Mechanical stimuli reportedly activate TRPV4 expressed in esophageal keratinocytes that in turn leads to increased ATP release[4]. To examine whether TRPV4 stimulation has a similar effect in gastric epithelium, we measured ATP release in chemically- or mechanically-stimulated RGE1-01 cells using a luciferin-luciferase assay. TRPV4 agonists GSK1016790A (GSK, 100 nmol/L) or 5,6-EET (500 nmol/L)[2] significantly increased ATP release in RGE1-01 cells (Figure 5A, 2- to 3-fold higher *vs* control, $P < 0.05$). Additionally, 120% lateral stretch applied for 3 min to RGE1-01 cells cultured on a silicon chamber induced significantly higher amounts of ATP release (Figure 5B, about 2-fold *vs* without stretch (control), $P < 0.05$), and these responses were inhibited by the specific TRPV4 inhibitor HC 067047 (1 μmol/L) (stretched cells showed no detachment over the 3-min stretch period). These results suggested that chemical and mechanical stimuli-induced ATP release in RGE1-01 cells was mediated by TRPV4 channel activation.

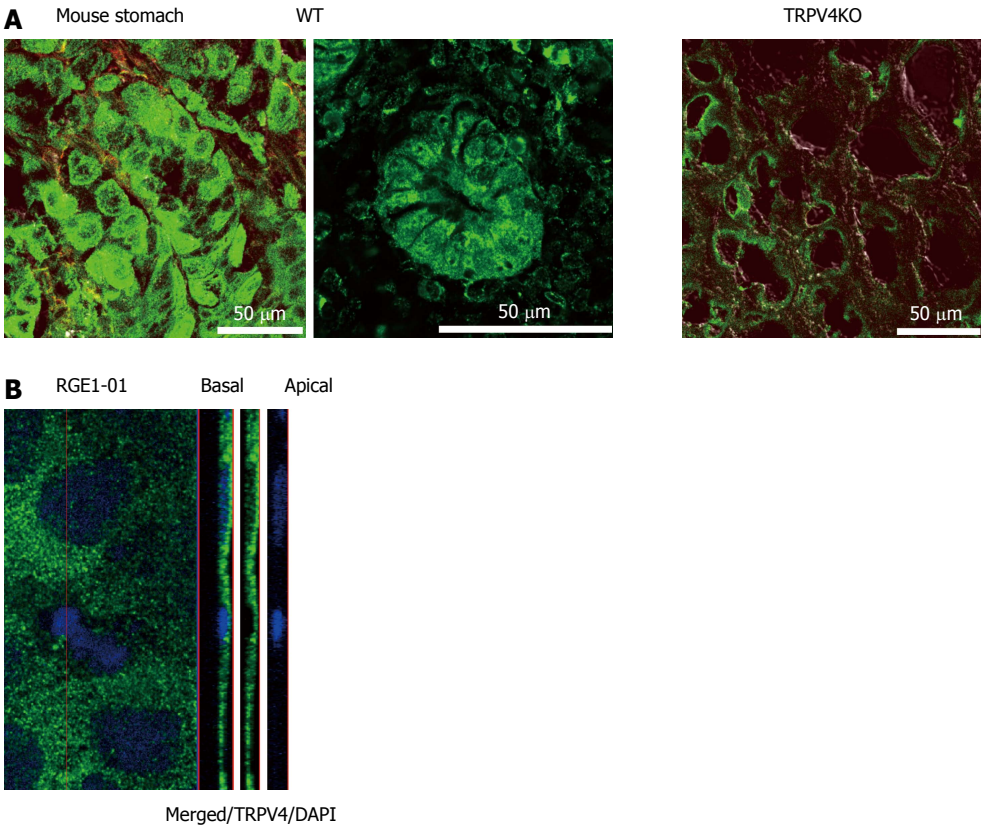

Figure 2 TRPV4 protein expression in mouse stomach and RGE1-01 cells. A: TRPV4 expression was homogeneously observed in WT but not TRPV4KO mouse gastric epithelium. Bars indicate 50 μm; B: Z-stack image of RGE1-01 cells demonstrated apical TRPV4 expression.

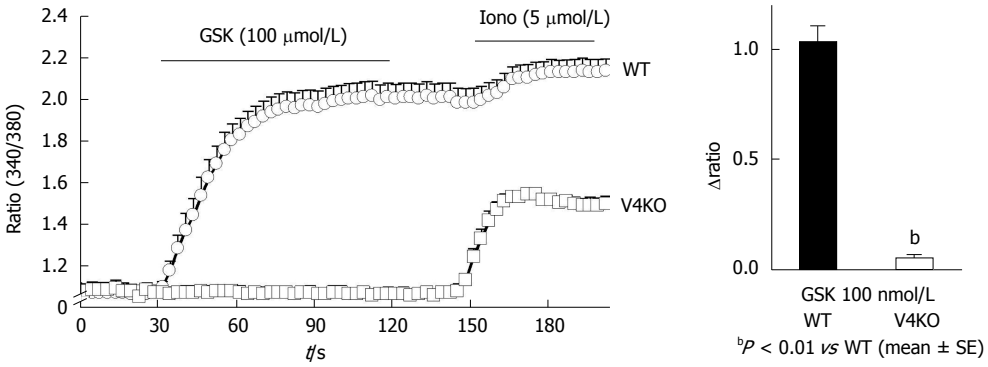

Figure 3 TRPV4-mediated increases in cytosolic Ca^{2+} ($[Ca^{2+}]_i$) in mouse primary gastric epithelial cells. A: $[Ca^{2+}]_i$ changes (340/380 ratio) in response to the TRPV4 specific agonist GSK1016790A (GSK, 100 nmol/L) in WT or TRPV4KO (V4KO) primary gastric epithelial cells (mean ± SEM). Ionomycin (iono, 5 μmol/L) was used as a positive control. Bars indicate the period of chemical application; B: GSK significantly increased $[Ca^{2+}]_i$ in WT cells (means ± SD; 1.03 ± 0.07, n = 20) compared to TRPV4KO cells (0.05 ± 0.01, n = 20) ($^bP < 0.01$ *vs* WT).

Delayed gastric emptying in TRPV4KO mice

Since TRPV4 has been shown to sense chemical and mechanical stimuli and contribute to ATP release from gastric epithelial cells, we hypothesized that TRPV4KO mice would exhibit altered gastric motility. To evaluate the physiological role of TRPV4 expressed in the gastric epithelium, we performed an *in vivo* experiment to compare gastric emptying rates of WT and TRPV4KO mice. Gastric emptying rates in TRPV4KO mice were about 2/3 of those in WT (Figure 6), suggesting that TRPV4 contributes to gastric motor function.

DISCUSSION

We identified morphological and functional TRPV4 expression in mouse gastric epithelial cells as well as the rat gastric epithelial cell line RGE1-01 (Figures 1-4). Furthermore, we demonstrated that chemical and mechanical stimuli can induce TRPV4-dependent ATP release from RGE1-01 cells (Figure 5), and that stimulation of gastric epithelial TRPV4 enhances gastric emptying *in vivo* (Figure 6). Using immunohistochemistry, Ca^{2+} imaging,

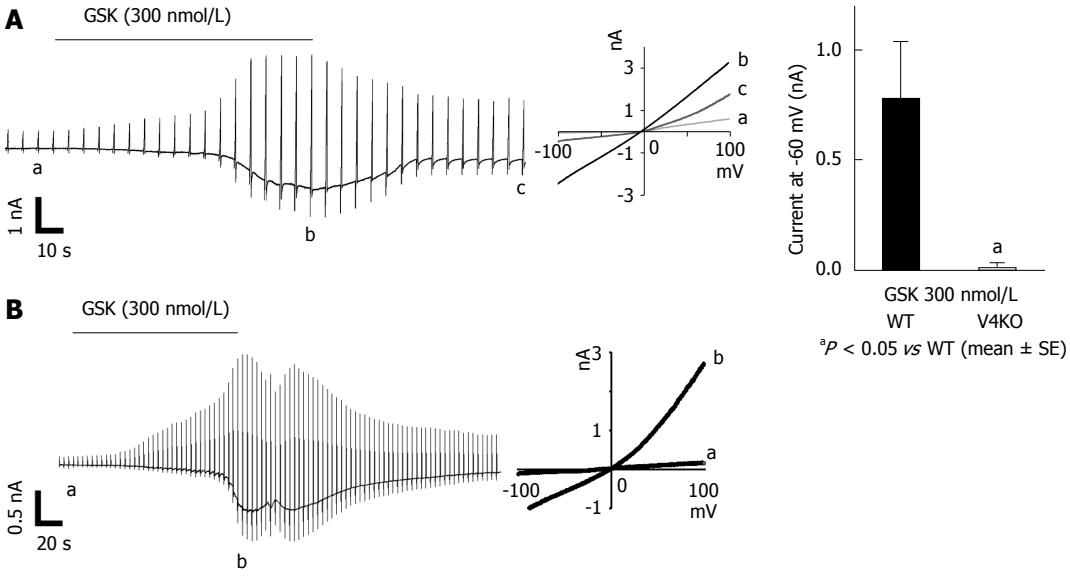

Figure 4 TRPV4-mediated current responses in mouse primary gastric epithelial cells and RGE1-01 cells. A: GSK (300 nmol/L) evoked inward current responses in WT primary gastric epithelial cells. Currents in response to ramp-pulses at points a, b and c (left in panel B) are shown (middle), with a strongly outwardly rectifying current-voltage relationship. Significantly larger inward currents at -60 mV were obtained from WT cells (means ± SEM; 0.76 ± 0.27 nA, n = 5) than in TRPV4KO cells (0.01 ± 0.00 nA, n = 5) ([a]P < 0.05 *vs* WT). B: Similar current responses were also obtained in RGE1-01 cells.

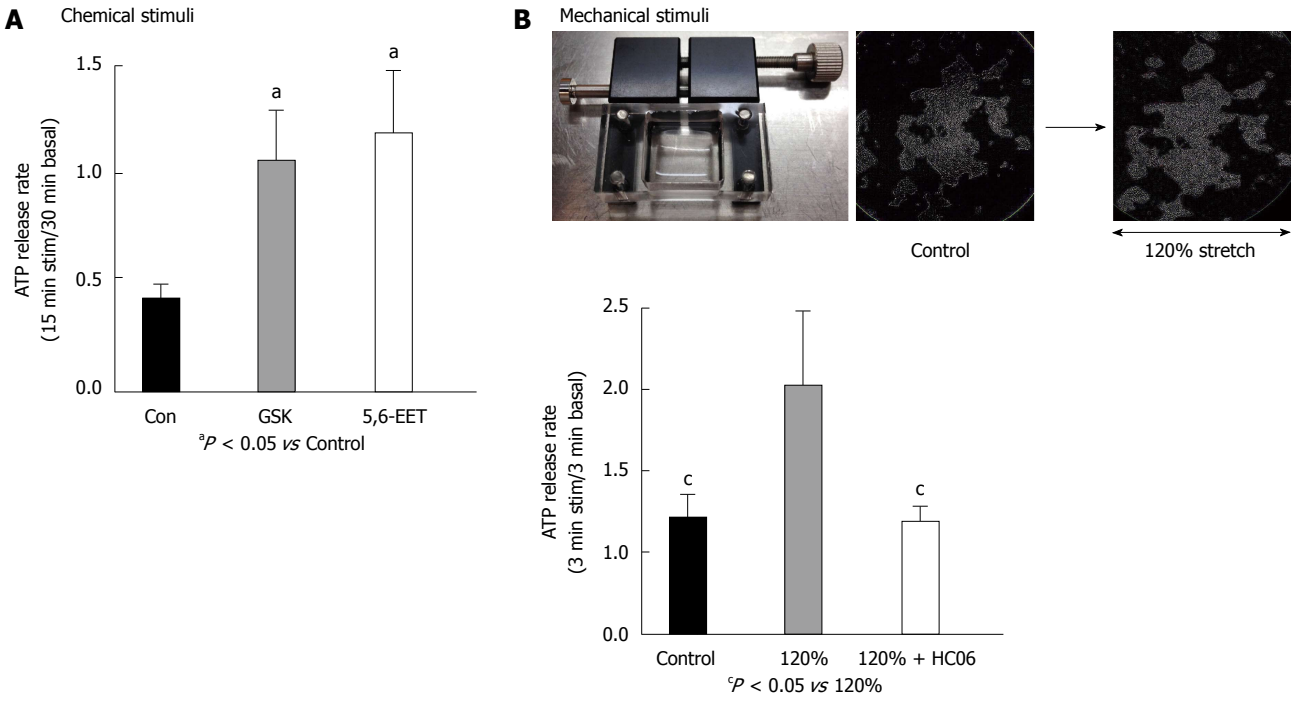

Figure 5 TRPV4 activator-induced ATP release in the RGE1-01 cells. A: GSK1016790A (GSK, 100 nmol/L) or 5,6-EET (500 nmol/L) induced significantly higher ATP release in RGE1-01 cells ([a]P < 0.05 *vs* Control). B: Mechanical stimuli were quantitatively applied with a stretch apparatus. Microscopy images demonstrated that cells were stretched laterally without detachment. A 120% stretch induced significantly higher amounts of ATP release from RGE1-01 cells [[c]P < 0.05 *vs* 0% stretch (control)] that could be inhibited by pre-treatment with specific TRPV4 antagonist HC 067047 (1 μmol/L). TRPV4: Transient receptor potential vanilloid 4.

and electrophysiology, we found that the majority of mouse gastric epithelial cells exhibited abundant TRPV4 expression and responded to TRPV4 agonists, suggesting that TRPV4 is a candidate mechanoreceptor in gastric epithelial cells. In fact, ATP release was several hundred nmol/L in our *in vitro* study, suggesting that the corresponding ATP concentration *in vivo* might be estimated to be several μmol/L, which would be sufficient to activate the P2 receptor present in the wall of mouse stomach[21,22]. These results suggested the hypothesis that luminal distension stimulates TRPV4 on gastric epithelial cells that in turn release ATP. The released ATP either stimulates subepithelial sensory nerve fibers that form the afferent limb of short or long gastrointestinal reflex arcs or acts directly on visceral smooth muscles expressing

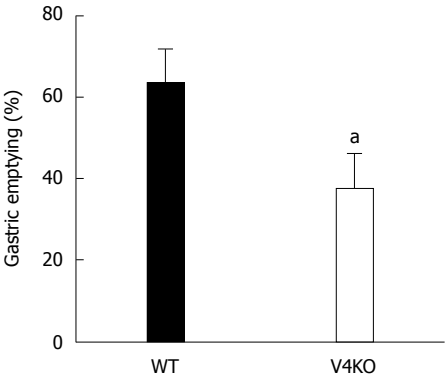

Figure 6 Delayed gastric empting in TRPV4 knockout mice. Gastric emptying rates *in vivo* in TRPV4KO mice were significantly delayed relative to WT mice ($^{a}P < 0.05$, *vs* WT, $n = 7$-9).

purinergic receptors. The first possibility is supported by morphological evidence showing that purinergic receptors, mainly P2X2 and P2X3, were identified on putative gastric mechanosensing structures, including the vagal afferent intraganglionic laminar endings that are located in close proximity to the epithelium[23,24]. These vagal afferents form the afferent limb of the central vago-vagal reflex (long reflex arc) and are known to increase gastric motility following stimulation[25]. The hypothesis is also supported by findings from a previous study wherein P2X3-knockout mice show a blunted neural response to gastric distension and no differences in distension-evoked ATP release between knockout and control mice[26]. The released ATP could also trigger a local reflex arc intrinsic to the stomach wall, which is supported by results from a previous study wherein ATP was shown to induce tetrodotoxin-sensitive contraction responses mediated by neuronal P2X receptors in an *in vitro* whole-stomach preparation[27].

The possibility that ATP released from gastric epithelium could directly stimulate purinergic receptors expressed on gastric visceral smooth muscles is rather unlikely considering the short half-life of ATP and the distance that ATP must cross while diffusing from the gastric epithelium to visceral smooth muscles. Moreover, gastric smooth muscles are known to functionally express P2Y receptors that mediate relaxation in response to ATP[27] and would be expected, upon stimulation, to delay gastric emptying, which is opposite to our current findings. This outcome further decreases the likelihood of a direct effect for ATP released from gastric epithelium on smooth muscles. However, the purinergic signaling pathway that mediates gastric distension-induced epithelial TRPV4 stimulation requires further future characterization.

In conclusion, TRPV4 is morphologically and functionally expressed in mouse and rat gastric epithelia and contributes to ATP release and gastric emptying. Our results suggest that TRPV4 could be a promising novel diagnostic and therapeutic target for functional gastrointestinal disorders.

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COMMENTS

Background

The transient receptor potential vanilloid 4 channel (TRPV4) is a non-selective cation channel that is activated by mechanical stimuli. ATP has been recognized as an important signaling molecule *via* cell surface ATP receptors. This study explored TRPV4 expression in mouse and rat stomach and whether the stimulation mediated ATP release.

Research frontiers

The authors have reported that ATP release in the esophagus and urothelium is mediated by TRPV4 stimulation.

Innovations and breakthroughs

This is the first study showing TRPV4 expression and ATP release by its stimulation in the mouse stomach and/or rat gastric epithelial cells.

Applications

These data suggested the hypothesis that luminal distension stimulates TRPV4 on gastric epithelial cells that in turn release ATP. However TRPV4 expression in human gastric epithelium and the purinergic signaling pathway requires further evaluation.

Terminology

TRPV4 is a non-selective cation channel, that is activated by several physical and chemical stimuli, including mechanical stimuli. The channel activation increases intracellular Ca^{2+} concentration and elicits whole-cell currents in TRPV4-expressing cells.

Peer-review

The manuscript describes an original research performed in mouse stomach and in rat epithelium cell line focusing on the expression and function of TRPV4 ion channels. The study concept is based on previous findings of the authors regarding TRPV4-mediated ATP release on the esophasgus. The series of experiments in this manuscript demonstrated delayed gastric emptying in TRPV4 knockout mice compared to their WT littermates, as well as TRPV4 expression in mRNA and protein levels in both mouse and rat gastric epithelial cell line. In general, the idea is interesting, the various morphological and functional methods are sophisticated, the figures are demonstrative.

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ORIGINAL ARTICLE

Basic Study

Intravoxel incoherent motion diffusion-weighted imaging for monitoring chemotherapeutic efficacy in gastric cancer

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Author contributions: Song XL and Jeong YY designed the studies; Song XL, Kang YJ and Moon CM performed the majority of experiments; Song XL, Ahn KY, Kang YJ and Cho HJ contributed to the analysis and interpretation of imaging date and histological examination; Song XL wrote the first draft of the manuscript; Jeong GW and Kang HK have approved the final manuscript and completed manuscript; Also, all authors agree with the content of the manuscript.

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Animal care and use statement: The animal protocol was designed to minimize pain or discomfort to the animals according to Institutional Animal Care and Use Committee Guidelines. All mice, fed with a standard diet with water ad libitum, were maintained at appropriate laboratory conditions (a photoperiod of 12 h light and darkness, 50% humidity, 23 ℃). After MRI examination, mice were euthanized by overdose of isoflurane (over than 5%) for tissue collection.

Conflict-of-interest statement: The authors declared that they have no conflicts of interest to this work.

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Abstract

AIM: To assess intravoxel incoherent motion diffusion-weighted imaging (IVIM-DWI) for monitoring early efficacy of chemotherapy in a human gastric cancer mouse model.

METHODS: IVIM-DWI was performed with 12 *b*-values (0-800 s/mm^2) in 25 human gastric cancer-bearing nude mice at baseline (day 0), and then they were randomly divided into control and 1-, 3-, 5- and 7-d treatment groups (n = 5 per group). The control group underwent longitudinal MRI scans at days 1, 3, 5 and 7, and the treatment groups underwent subsequent MRI scans after a specified 5-fluorouracil/calcium

folinate treatment. Together with tumor volumes (TV), the apparent diffusion coefficient (ADC) and IVIM parameters [true water molecular diffusion coefficient (D), perfusion fraction (f) and pseudo-related diffusion coefficient (D^*)] were measured. The differences in those parameters from baseline to each measurement (ΔTV%, ΔADC%, ΔD%, Δf% and ΔD^*%) were calculated. After image acquisition, tumor necrosis, microvessel density (MVD) and cellular apoptosis were evaluated by hematoxylin-eosin (HE), CD31 and terminal-deoxynucleotidyl transferase mediated nick end labeling (TUNEL) staining respectively, to confirm the imaging findings. Mann-Whitney test and Spearman's correlation coefficient analysis were performed.

RESULTS: The observed relative volume increase (ΔTV%) in the treatment group were significantly smaller than those in the control group at day 5 ($\Delta TV_{treatment}$% = 19.63% ± 3.01% and $\Delta TV_{control}$% = 83.60% ± 14.87%, P = 0.008) and day 7 ($\Delta TV_{treatment}$% = 29.07% ± 10.01% and $\Delta TV_{control}$% = 177.06% ± 63.00%, P = 0.008). The difference in ΔTV% between the treatment and the control groups was not significant at days 1 and 3 after a short duration of treatment. Increases in ADC in the treatment group ($\Delta ADC\%_{treatment}$, median, 30.10% ± 18.32%, 36.11% ± 21.82%, 45.22% ± 24.36%) were significantly higher compared with the control group ($\Delta ADC\%_{control}$, median, 4.98% ± 3.39%, 6.26% ± 3.08%, 9.24% ± 6.33%) at days 3, 5 and 7 (P = 0.008, P = 0.016, P = 0.008, respectively). Increases in D in the treatment group ($\Delta D\%_{treatment}$, median 17.12% ± 8.20%, 24.16% ± 16.87%, 38.54% ± 19.36%) were higher than those in the control group ($\Delta D\%_{control}$, median -0.13% ± 4.23%, 5.89% ± 4.56%, 5.54% ± 4.44%) at days 1, 3, and 5 (P = 0.032, P = 0.008, P = 0.016, respectively). Relative changes in f were significantly lower in the treatment group compared with the control group at days 1, 3, 5 and 7 follow-up (median, -34.13% ± 16.61% *vs* 1.68% ± 3.40%, P = 0.016; -50.64% ± 6.82% *vs* 3.01% ± 6.50%, P = 0.008; -49.93% ± 6.05% *vs* 0.97% ± 4.38%, P = 0.008, and -46.22% ± 7.75% *vs* 8.14% ± 6.75%, P = 0.008, respectively). D* in the treatment group decreased significantly compared to those in the control group at all time points (median, -32.10% ± 12.22% *vs* 1.85% ± 5.54%, P = 0.008; -44.14% ± 14.83% *vs* 2.29% ± 10.38%, P = 0.008; -59.06% ± 19.10% *vs* 3.86% ± 5.10%, P = 0.008 and -47.20% ± 20.48% *vs* 7.13% ± 9.88%, P = 0.016, respectively). Furthermore, histopathologic findings showed positive correlations with ADC and D and tumor necrosis (r_s = 0.720, P < 0.001; r_s = 0.522, P = 0.007, respectively). The cellular apoptosis of the tumor also showed positive correlations with ADC and D (r_s = 0.626, P = 0.001; r_s = 0.542, P = 0.005, respectively). Perfusion-related parameters (f and D^*) were positively correlated to MVD (r_s = 0.618, P = 0.001; r_s = 0.538, P = 0.006, respectively), and negatively correlated to cellular apoptosis of the tumor (r_s = -0.550, P = 0.004; r_s = -0.692, P < 0.001, respectively).

CONCLUSION: IVIM-DWI is potentially useful for predicting the early efficacy of chemotherapy in a human gastric cancer mouse model.

Key words: Gastric cancer; Microvessel density; Nude mouse model; Intravoxel incoherent motion diffusion-weighted imaging; Terminal-deoxynucleoitidyl transferase mediated nick end labeling

Core tip: Intravoxel incoherent motion diffusion-weighted imaging (IVIM-DWI) is useful for monitoring changes of molecular diffusion and microcirculation in gastric cancer at the early stage of chemotherapy. The apparent diffusion coefficient (ADC) and IVIM parameters of true water molecular diffusion coefficient (D) could be reliable marker to detect the necrosis and cellular apoptosis, while perfusion-related IVIM parameters of perfusion fraction (f) and pseudo-related diffusion coefficient (D^*) are capable of noninvasive assessment of angiogenesis activity in gastric cancer undergoing chemotherapy.

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INTRODUCTION

Gastric cancer (GC) remains the fourth most common malignancy and the second-leading cause of cancer deaths in the world[1]. Despite many advances in cancer diagnosis and treatment, approximately two-thirds of patients are diagnosed with advanced gastric cancer (AGC) in many countries, excluding some trial results in Japanese and South Korean patients[2,3]. For many years, 5-fluorouracil (5-FU)-based chemotherapy has been considered a standard chemotherapy regimen for AGC[2-4]. Unfortunately, not all patients benefit from this regimen. If an ineffective therapy can be identified at an early stage of treatment, there will be an opportunity to change the therapy approach and clinical management in the individual patient quickly. The response evaluation criteria in solid tumors (RECIST) is widely adopted standard for evaluating therapy response based on the change in tumor size in clinical practice. However, this often takes several weeks to months to develop, and its evaluation period is too long to adjust patient management[5]. Therefore, increasing demands are being placed on imaging modalities to identify early

and reliable surrogate markers for the evaluation of therapeutic effect in patients with GC[6].

Diffusion-weighted magnetic resonance imaging (DWI) is capable of providing an apparent diffusion coefficient (ADC), which is a measure magnitude of diffusion (of water molecules) within tissue, and has become a favorite choice for oncologic studies[7]. It is well known that a lower ADC is a characteristic of most tumors compare with native tissues because of their high cellularity. An increase in tissue diffusivity, which is induced by an enlarged extracellular space, cell swelling or a loss of membrane integrity under an effective therapy, makes it possible to use ADC to identify the treatment response[8-11]. However, the ADC value derived from DWI based a mono-exponential model does not sufficiently demonstrate the characteristics of tissue behavior. The *b*-value, which represents the strength and timing of the gradients, determines DWI sensitivity to water motion. When a lower *b*-value is applied ($\leqslant$ 100 s/mm^2), microcirculation related protons have relative large diffusion distances, and are capable of changing diffusion signal intensities. In 1986, Le Bihan *et al*[12] first described the concept of intravoxel incoherent motion (IVIM), which can be used to estimate molecular diffusion and microcirculation in the capillaries separately through bi-exponential fitting of the DWI data using multiple *b*-values. In recent years, there has been increasing interests in IVIM-DWI, which allows acquisition of quantitative parameters that reflect tissue diffusivity and microcirculation perfusion simultaneously. The true water molecular diffusion coefficient (D), perfusion fractional (f) volume reflective of capillary blood volume and pseudo-related diffusion coefficient associated with capillary network blood flow (D*) can be measured using IVIM-DWI. The blood microcirculation within capillaries can be considered as a type of "pseudo-diffusion" because it has no specific orientation[13].

It has been determined that IVIM-DWI can provide a new opportunity to gain an insight into the perfusion of a tumor without contrast agent administration for preclinical and clinical applications[12,14,15]. Koh *et al*[16] reported that the calculated f values were lower in colorectal liver metastases, which are characterized by their hypovascular nature, than in the normal liver. In addition, DWI with 10 *b*-values between 0 and 700 s/mm^2 enables the measurement of diffusion and microcirculation contributions in renal allografts as early as 5-d after transplantation[17]. Moreover, IVIM-DWI has been used to evaluate the treatment responses to radiofrequency ablation[18] or a vascular disrupting agent of CKD-516[19] in rabbit model with VX2 liver tumors, and to neoadjuvant chemotherapy in human locoregionally advanced nasopharyngeal carcinoma. Although DWI is increasingly being applied in the body, few studies have focused on gastric lesions due to the limitations of modality of the gastric[20]. Recently, Cheng *et al*[21] applied IVIM with *b*-values of up to 1500 s/mm^2 to evaluate chemotherapeutic efficacy in a gastric cancer xenograft model. In that study, f increased after chemotherapy treatment, which refutes currents theories regarding angiogenesis activity within tumors after treatment[22]. A previous IVIM study in prostate cancer also found that f in tumors significantly increased compared to that in normal prostate tissue when a *b*-value of less than 750 s/mm^2 was used, while f decrease or became indistinguishable from the normal prostate tissue when high *b*-values were employed[23]. This phenomenon could be explained by the following theory: The departure of molecular diffusion at very high *b*-values may have an influence on perfusion-related parameters because both water diffusion and microcirculation contribute to the signal attenuation observed at lower *b*-values ($\leqslant$ 100 s/mm^2)[13,15]. It should be noted that higher b values may affect the accuracy of the IVIM-derived parameters, which depend heavily on the *b*-value selection.

Here, we investigate IVIM-DWI with *b*-values below 800 s/mm^2 as a potential imaging marker for assessing the early chemotherapy response in term of tissue diffusion and microvascular perfusion, by comparing the tissue cellularity and microvascular density (MVD) properties revealed by histopathological analysis during the full course of treatment using a mouse human gastric cancer xenograft model.

MATERIALS AND METHODS

Animal model

This study included 25 male adult (6 weeks old) nude mice (BALB/c-nu/nu, Orient Bio, Gwangju, South Korea), each weighing 20-25 g. All mice, fed with a standard diet with water ad libitum, were maintained in appropriate laboratory conditions (a photoperiod of 12 h light and darkness, 50% humidity, 23 ℃). After a two-week adaption period, 1 × 10^7 human gastric adenocarcinoma AGS cells (ATCC®, CRL-1739™) suspended in 100 μL cold PBS were subcutaneously injected into the lower right hind limbs of the nude mice with a 31 gauge syringe. To evaluate the therapeutic response, the tumor growth curves in each group were estimated based on the morphologic T2-weighted images by using the following formula: TV = π/6 × L × W × H (L, length, W, width, H, height of the tumor).

Study design

Following a baseline (day 0) MRI examination, human gastric cancer-bearing mice were randomly divided into control and 1-d, 3-d, 5-d, and 7-d treatment groups (n = 5 per group). 5-fluorouraci (5-FU) (15 mg/kg)/calcium folinate (5 mg/kg) was used for chemotherapy in the treatment groups, while same volume of saline was administered in the control group. 5-FU/calcium folinate was intraperitoneally injected on a bi-daily basis. Mice in the control group underwent longitudinal MRI at days 1, 3, 5 and 7. Each

treatment group underwent a second scan with same MRI protocol at days 1, 3, 5, or 7 after treatment. After the MRI examination, mice were euthanized by an overdose of isoflurane, and the tumor was stripped for further analysis. The short-term 7-d 5-FU treatment was performed, because this study was aimed to investigate the potential of IVIM-DWI for monitoring the early tumor diffusion and perfusion response to treatment.

MRI protocol

All MRI scans were performed using 3T MRI (GE Healthcare, Waukesha, WI, United States) with a wrist coil. Mice were placed on a heated pad and anesthetized with 2% isoflurane in oxygen (at a rate of 1.0 L/min) to void movement during imaging. After acquisition of the routine images for localization, a transverse T2-weighted image was obtained using fast spin echo (FSE) sequence [repetition time/echo time (TR/TE), 2000/99.6 ms; section thickness, 3 mm; matrix, 512 × 358; number of excitations (NEX), 4]. Subsequently, IVIM-DWI with 12 *b*-values (0, 10, 15, 20, 25, 30, 60, 75, 100, 200, 400 and 800 s/mm^2) were acquired using a free-breathing single-shot echo-planar imaging (EPI) sequence with application of three diffusion-gradients directions (TR/TE, 2500/66.5; section thickness, 3 mm; number of sections, 8; field of view, 10 × 10 cm^2; matrix, 128 × 128; NEX, 4) with an acquisition time of 7 min and 30 s for each study.

MR imaging analysis

The acquired datasets were transferred to a GE workstation (Advance Workstation 4.6) and analyzed using an in-house software. The ADC value was calculated by using a liner fit (least-squares fit) mono-exponential model based on the following equation: $S_b/S_0 = \exp(-b \times ADC)$[15], where S_0 is the signal intensity at a *b* value of 0 and S_b is the signal intensity at higher *b* values. IVIM parameters was calculated by a nonlinear fit (Levenberg-Marquardt fit)bi-exponential model, and the equation is shown as follow: $S_b/S_0 = (1\text{-}f) \times \exp(-bD) + f \times \exp[-b(D + D^*)]$[15], where D represents true water molecular diffusion coefficient, f and D* represent perfusion fraction and pseudo-related diffusion coefficient, respectively. A technician with 8 years of experience in MRI measured the tumor sizes and values of ADC, D, f, and D*. The technician was blinded to the information regarding the treatment and control groups.

Regions of interest (ROIs) were drawn by outlining the tumor border on ADC maps, which showed the largest cross-section of the tumor. The ROIs on ADC map were copied and pasted on the corresponding D, f, and D* maps. In each mouse, the change in tumor volume (TV) relative to the baseline was quantified to determine the treatment response as follows: $\Delta TV\% = [(TV_{given\ time} - TV_{baseline})/TV_{baseline}] \times 100$, where $TV_{given\ time}$ is the tumor volume on day 1, 3, 5, or 7 and $TV_{baseline}$ is the tumor volume on day 0. For the ADC and IVIM parameters, the percentage changes in values compared to the baseline were recorded by using the following formula: $\Delta Value\% = [(Value_{given\ time} - Value_{baseline})/Value_{baseline}] \times 100$, where $value_{given\ time}$ is the value on day 1, 3, 5, or 7 and $Value_{baseline}$ is the value on day 0.

Histopathological analysis

The tumor tissue was paraffin-embedded, and sliced in the transverse plane at 0.6 μm intervals to match the corresponding MR image, was selected. The necrotic fraction (NF) of the tumor was evaluated through hematoxylin-eosin (HE) staining. CD31 (Dako, Carpinteria, CA, United States; mouse anti-human; used at 1:200) staining was carried out on pathological specimens by immunohistochemical methods, to analyze the angiogenesis activity of the tumor. The paraffin-embedded sections were incubated with the primary antibody at 4 ℃ overnight. Secondary antibody (Stem cell Technologies; rabbit anti-mouse; used at 1:300) was applied at room temperature for 1 h and then sections were rinsed with PBS. Positive reaction was visualized by DAB chromogen (Dako, Carpinteria, CA, United States) according to standard methods. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (Roche Applied Science, Penzberg, Germany) was performed to evaluate cellular apoptosis of the tumor by following the manufacturer's instructions.

Histopathological analysis was performed by using Image J software (http://rsb.info.nih.gov/ij). Tumor necrosis was scored according to the following formula: $NF = Area_{necrosis}/Area_{total\ tumor}$. Cellular apoptosis of the tumor was defined as the percentage of positive TUNEL-stained cells among 200 nuclei from five randomly selected fields at a high magnification (× 200). The mean MVD of the tumor was defined by the CD31-stained vessels[24], where any distinct area of positive for CD31 staining was defined as a single vessel, from five hot spots with higher vascular density compared to the remaining tissue in a high-power field (× 200; 0.578 mm^2).

Statistical analysis

SPSS 21.0 (SPSS, Chicago, IL, United States) was used for statistical analysis. The differences in relative changes in the ADC and IVIM parameters, and tumor volumes between the control and treatment groups were determined by the Mann-Whitney test. Spearman's correlation coefficient was used to determine the correlations between histological features, including NF, MVD and TUNEL and the corresponding ADC and IVIM parameters. Spearman's coefficient was considered to be satisfactory with a critical value of $r_s = 0.415$ at P value < 0.05 (two-tailed test). A spearman's coefficient of 0.90-1.00 indicated almost perfect agreement; 0.70-0.90 indicated high

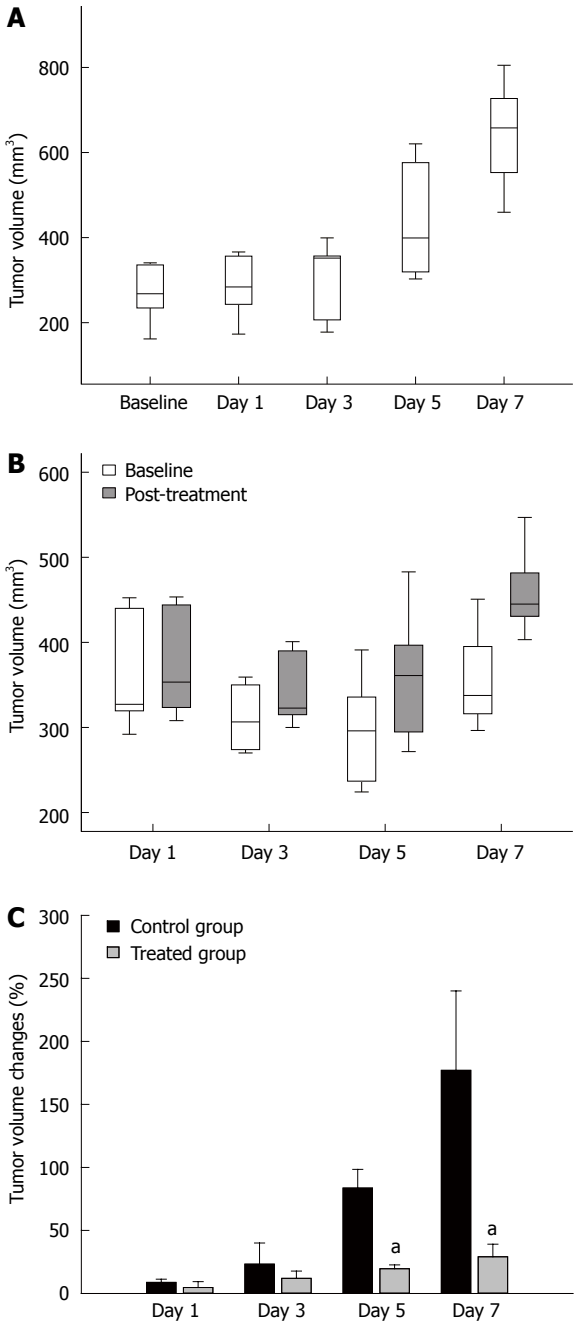


Figure 1 Anti-tumor effects of 5-fluorouracil therapy in mouse gastric cancer xenografts. A: Actual tumor volume changes in the control group; B: Actual tumor volume changes in the treatment groups; C: The comparison of the change in tumor volumes relative to baseline (day 0) between the control and treatment groups. The control group showed increases in tumor volume, while the treatment group showed as significant tumor growth delay from day 5. Center line = median; upper and lower margins of the box = 25th to the 75th percentile, respectively; whiskers = data from the minimum to the maximum. Error bars denote standard errors. $^{a}P < 0.05$ *vs* control group, $n = 5$ in each group.

agreement; 0.50-0.70 indicate moderate agreement; 0.30-0.50 indicate low agreement; and 0.00-0.30 indicate negligible agreement[25]. A two-tailed *P* value < 0.05 was considered as significant difference.

RESULTS

Effects of chemotherapy on tumor growth

Twenty-five tumors with a mean volume of 298.07 ± 103.44 mm^3 (range: 194.32-432.34 mm^3) before treatment were analyzed 20 to 25 d of implantation. The tumor volume in the control and treatment groups is shown in Figure 1A and B. 5-FU induced a significant growth delay, as assessed by the MRI-derived tumor volume measurements in comparison with that in the control group from day 5. As shown in Figure 1C, the observed relative volume increase (ΔTV%) in the therapy groups were significantly lower than in the control group at day 5 ($\Delta TV_{treatment}\%$ = 19.63% ± 3.01% and $\Delta TV_{control}\%$ = 83.60% ± 14.87%, $P = 0.008$) and day 7 ($\Delta TV_{treatment}\%$ = 29.07% ± 10.01% and $\Delta TV_{control}\%$ = 177.06% ± 63.00%, $P = 0.008$). The difference in ΔTV% between the treatment and the control groups was not significant at day 1 ($\Delta TV_{treatment}\%$ = 4.97% ± 4.59% and $\Delta TV_{control}\%$ = 8.08% ± 2.47%, $P = 0.841$) or day 3 ($\Delta TV_{treatment}\%$ = 15.36% ± 5.75% and $\Delta TV_{control}\%$ = 23.28% ± 16.76%, $P = 0.310$) after a short treatment duration.

IVIM-DWI assessment of a human gastric cancer xenograft

Table 1 summarizes the ADC, D, f, and D* values of the tumors in the control and treatment groups, that were measured at baseline and at days 1, 3, 5 and 7. Figure 2 shows the mean percentage changes in the DWI parameters relative to baseline at each time point in each group. In the control group, all ADC values and IVIM parameters of the tumor remained relatively constant over the 7-d experiment. ADC increases in the treatment group ($\Delta ADC\%_{treatment}$, median, 30.10% ± 18.32%, 36.11% ± 21.82%, 45.22% ± 24.36%) were significantly higher compared with the control group ($\Delta ADC\%_{control}$, median, 4.98% ± 3.39%, 6.26% ± 3.08%, 9.24% ± 6.33%) at days 3, 5 and 7 ($P = 0.008$, $P = 0.016$, $P = 0.008$, respectively) (Figure 2A). Increases in D in the treatment group ($\Delta D\%_{treatment}$, median 17.12% ± 8.20%, 24.16% ± 16.87%, 38.54% ± 19.36%) were higher than in the control group ($\Delta D\%_{control}$, median -0.13% ± 4.23%, 5.89% ± 4.56%, 5.54% ± 4.44%) at days 1, 3, and 5 ($P = 0.032$, $P = 0.008$, $P = 0.016$, respectively) (Figure 2B). The relative changes in f were significantly lower in the treatment group than in the control group at days 1, 3, 5 and 7 follow-up (median, -34.13% ± 16.61% *vs* 1.68% ± 3.40%, $P = 0.016$; -50.64% ± 6.82% *vs* 3.01% ± 6.50%, $P = 0.008$; -49.93% ± 6.05% *vs* 0.97% ± 4.38%, $P = 0.008$, and -46.22% ± 7.75% *vs* 8.14% ± 6.75%, $P = 0.008$, respectively) (Figure 2C). D* in the treatment group decreased significantly compared with the control group at all time points (median, -32.10% ± 12.22% *vs* 1.85% ±

Table 1 Summary of the apparent diffusion coefficient and intravoxel incoherent motion parameters at baseline (day 0) and on days 1, 3, 5, and 7 in each groups (n = 5 per group)

	ADC (10^{-3} mm^2/s)	IVIM parameters		
		D (10^{-3} mm^2/s)	f (%)	D^* (mm^2/s)
Control group				
Day 0	0.514 ± 0.050	0.480 ± 0.049	32.424 ± 6.647	0.112 ± 0.017
Day 1	0.520 ± 0.056	0.497 ± 0.052	33.010 ± 7.121	0.114 ± 0.016
Day 3	0.537 ± 0.029	0.506 ± 0.025	33.606 ± 8.113	0.115 ± 0.020
Day 5	0.545 ± 0.047	0.504 ± 0.021	32.602 ± 6.684	0.117 ± 0.018
Day 7	0.561 ± 0.053	0.524 ± 0.064	35.100 ± 7.858	0.120 ± 0.016
1-d treatment group				
Day 0	0.555 ± 0.028	0.524 ± 0.028	34.076 ± 7.247	0.171 ± 0.049
Day 1	0.597 ± 0.064	0.612 ± 0.065	21.912 ± 5.032	0.115 ± 0.040
3-d treatment group				
Day 0	0.528 ± 0.012	0.510 ± 0.019	37.066 ± 7.331	0.147 ± 0.025
Day 3	0.686 ± 0.107	0.636 ± 0.121	18.596 ± 5.766	0.083 ± 0.029
5-d treatment group				
Day 0	0.594 ± 0.069	0.567 ± 0.065	34.664 ± 8.337	0.173 ± 0.068
Day 5	0.804 ± 0.136	0.777 ± 0.107	17.344 ± 4.352	0.070 ± 0.045
7-d treatment group				
Day 0	0.560 ± 0.060	0.535 ± 0.081	37.598 ± 5.852	0.148 ± 0.082
Day 7	0.823 ± 0.221	0.710 ± 0.236	20.040 ± 3.042	0.065 ± 0.018

ADC: Apparent diffusion coefficient; IVIM: Intravoxel incoherent motion.

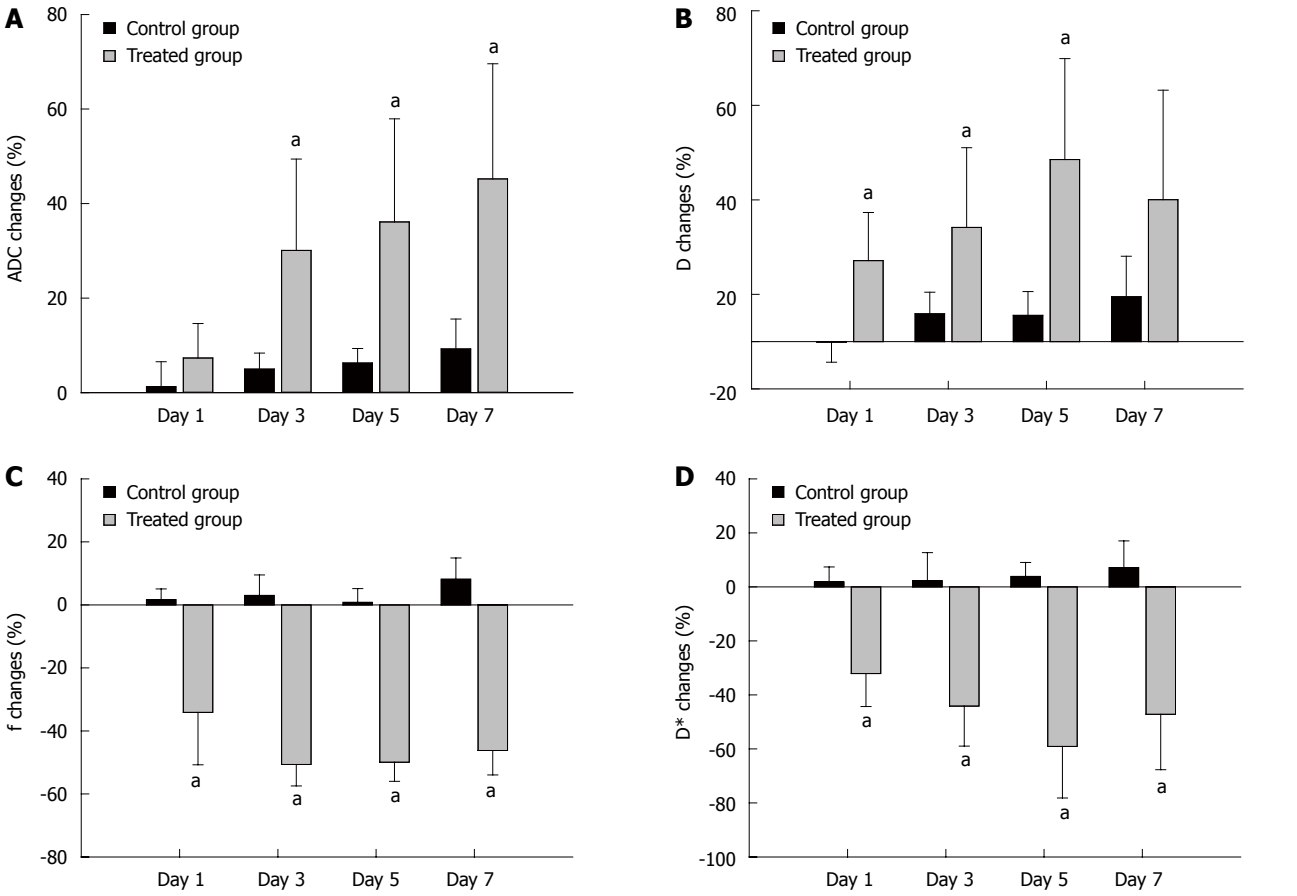


Figure 2 Comparison of the mean percentage changes from baseline in the intravoxel incoherent motion diffusion-weighted imaging derived values between the control (dark) and the 1-, 3-, 5- and 7-d treatment groups (grey). A: ADC value; B: D value; C: f value; D: D* value. Standard deviations are represented by vertical bars. Relative changes were determined by comparing the values at baseline and those in follow-up. $^aP < 0.05$ *vs* control. n = 5 in each group. ADC: Apparent diffusion coefficient.

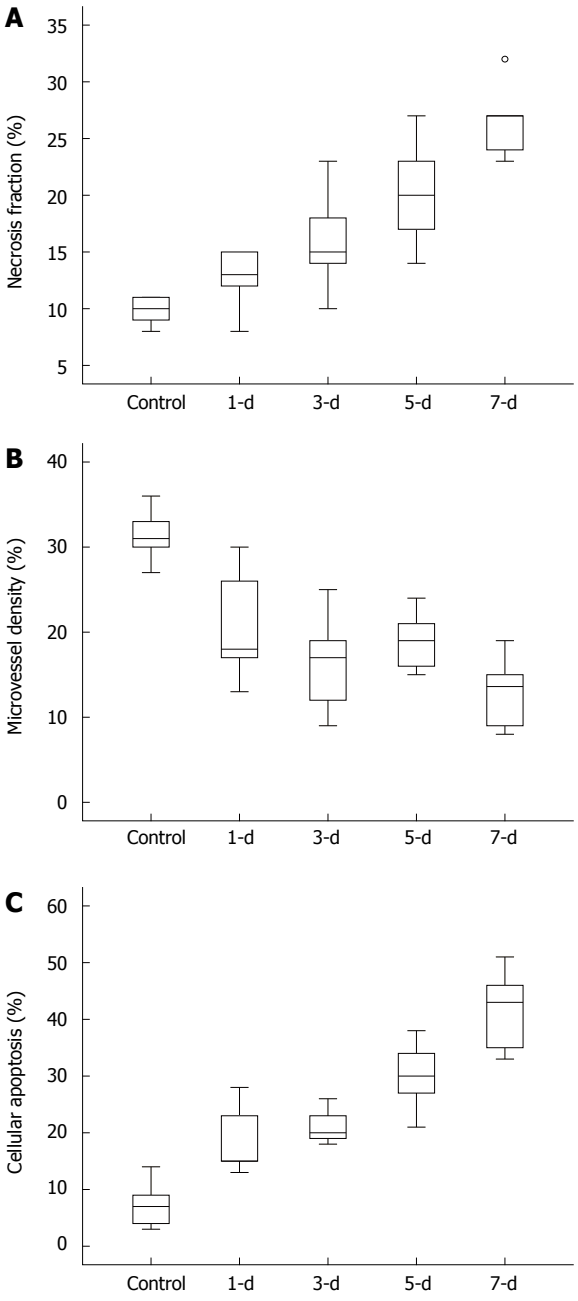


Figure 3 Box-and-Whisker plots show the results of histopathological analysis in the control and the 1-, 3-, 5- and 7-d treatment groups, respectively (*n* = 5 per group). A: Necrosis fraction of tumor; B: Microvessel density of tumor; C: Cellular apoptosis of tumor. Center line = median; upper and lower margins of box = 25th to the 75th percentile, respectively; whiskers = data from the minimum to the maximum; ○ = outlier.

5.54%, P = 0.008; -44.14% ± 14.83% *vs* 2.29% ± 10.38%, P = 0.008; -59.06% ± 19.10% *vs* 3.86% ± 5.10%, P = 0.008, and -47.20% ± 20.48% *vs* 7.13% ± 9.88%, P = 0.016, respectively) (Figure 2D).

Histopathological assessment of tumor response and its correlation with MR images

HE, CD31 and TUNEL staining were performed to confirm the tissue and vessel changes in the tumor. Figure 3 shows the quantification of the NF, MVD, and cellular apoptosis in all animals of the control and the treatment groups (n = 5 per group). The MVD scores in 5-FU treatment tumors decreased significantly compared with the control group. The 5-FU treated tumors displayed a time-dependent increase in NF and cellular apoptosis compared to those in the control group, indicating an effective therapeutic response.

Table 2 summarizes the relationship among ADC, the IVIM parameters, MVD, cellular apoptosis and necrosis of tumors (n = 25) determined by Spearman's correlation coefficient analysis. ADC and D were positively correlated with NF (r_s = 0.720, P < 0.001; r_s = 0.522, P = 0.007, respectively) (Figure 4A, B) and the cellular apoptosis of the tumor (r_s = 0.626, P = 0.001; r_s = 0.542, P = 0.005, respectively) (Figure 4E, F).There is no significant correlation among ADC, D, and MVD. Perfusion-related parameters f and D* shows positive correlations with MVD (r_s = 0.618, P = 0.001; r_s = 0.538, P = 0.006, respectively) (Figure 4C, D), and a negative correlation with the cellular apoptosis of the tumor (r_s = -0.550, P = 0.004; r_s = -0.692, P < 0.001, respectively) (Figure 4G, H). There is no significant correlation between the perfusion parameters and NF. Figure 5 shows the calculated ADC, D, f, and D* maps and the correspondent histopathologic images from the control and 3 d treated mice.

DISCUSSION

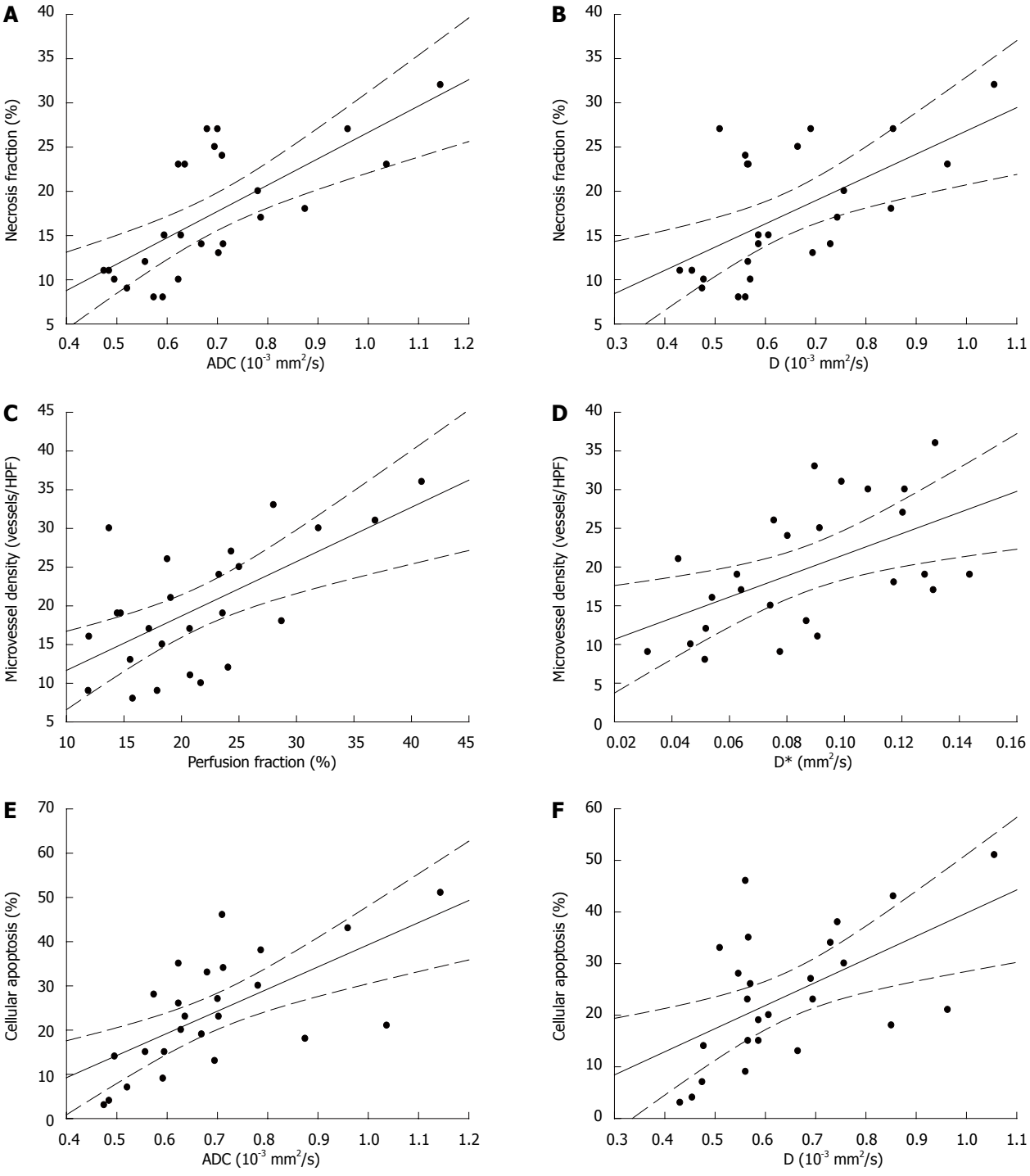
The ADC obtained from conventional DWI has been widely accepted as a marker to monitor the therapeutic efficacy of chemotherapy, radiotherapy or combined therapy with target medicine[26,27]. In recent years, there has been a resurgent interest in IVIM studies, which allows to measure tissue diffusion and perfusion simultaneously. In this study, we performed IVIM-DWI using 12 *b*-values less than 800 s/mm^2 to monitor the efficacy of chemotherapy in a mouse model of human gastric cancer. A histopathological analysis was carried out to evaluate the tissue cellularity and MVD properties of the tumor.

Our results demonstrated that conventional ADC significantly increased after 3-d of treatment and it showed a positive correlation with the 5-FU induced intratumoral necrosis and cellular apoptosis of the tumor. Papaevangelou *et al*[28] found that ADC changes in the tumor were associated with the induction of a mixture of necrosis and apoptosis after irinotecan treatment. Therefore, ADC could be a reliable marker for detecting the necrotic and apoptotic cell death in gastric cancer patients during treatment. IVIM derived D values that showed a similar trend to ADC, were significantly increased as early as after one day treatment compared with the baseline values. Moreover, HE and TUNEL stain showed that D values were positively correlated with intratumoral necrosis and cellular apoptosis, indicating the possibility of

Table 2 Correlation between apparent diffusion coefficient and intravoxel incoherent motion parameters, microvessel density, cellular apoptosis and necrosis fraction of tumors (n = 25)

	NF (%)		MVD (vessels/HPF)		Apoptosis (%)	
	r_s	*P* value	r_s	*P* value	r_s	*P* value
ADC ($\times 10^{-3}$ mm^2/s)	0.720	< 0.001	-0.395	0.051	0.626	0.001
IVIM parameters						
D ($\times 10^{-3}$ mm^2/s)	0.522	0.007	-0.201	0.335	0.542	0.005
f (%)	-0.347	0.089	0.618	0.001	-0.550	0.004
D^* ($\times$ mm^2/s)	-0.378	0.062	0.538	0.006	-0.692	< 0.001

P < 0.05 is considered to be statistically significant. ADC: Apparent diffusion coefficient; IVIM: Intravoxel incoherent motion; MVD: Microvessel density; NF: Necrosis fraction.



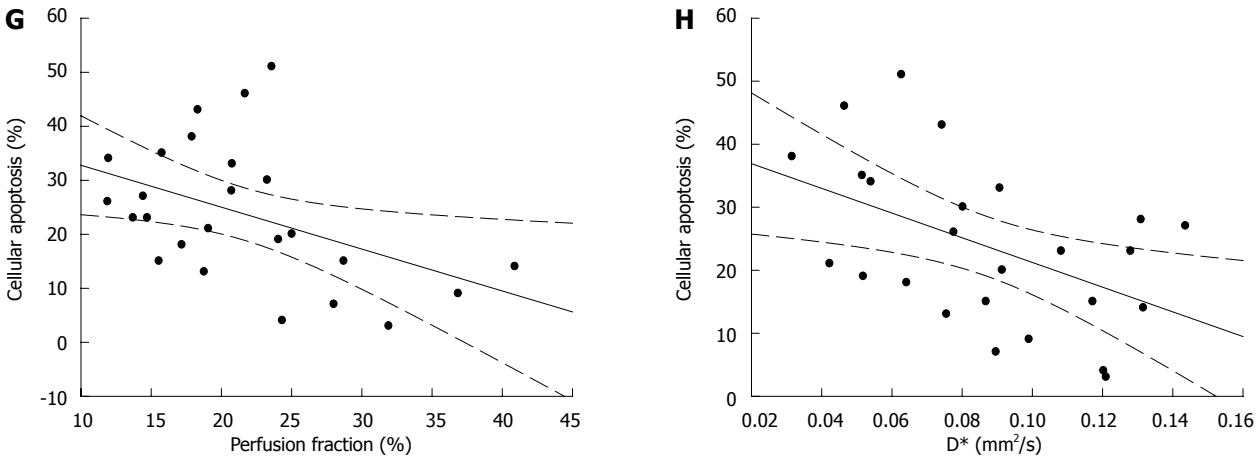


Figure 4 Representative scatter diagrams showing the relationships between intravoxel incoherent motion diffusion-weighted imaging derived parameters and the histological features (*n* = 25). A and B: ADC and D were positively correlated with tumor necrosis fraction; C and D: f and D* were positively correlated with microvascular density of tumor; E and F: ADC, D were positively correlated with tumor cellular apoptosis; G and H: f and D* were negatively correlated with tumor cellular apoptosis. *P* values are shown in Table 2. ADC: Apparent diffusion coefficient.

a noninvasive evaluation of necrosis or apoptotic. Our finding regarding the D value is consistent with previous studies which showed that an increase in D value can predict chemotherapeutic responsiveness in locoregionally advanced nasopharyngeal carcinoma[20] and advanced cervical cancer[29]. There is no denying that accurate quantification of the D value, which associates with the intra-to-extracellular spaces ratio and represents the true molecular diffusion, would eventually translate into a reliable marker to evaluate the tumor therapy response.

The f and D* significantly decreased during the early phase of chemotherapy. In addition, the f and D* were well correlated with the decrease in MVD revealed by the endothelial cell marker CD31 staining, which indicated that f and D* have the potential to assess tumor angiogenesis activity noninvasively. There are experimental and clinical dates in body tissues with IVIM-DWI which support the claim that the signal attenuation is related to microcirculation in tissue when *b*-values less than 100 s/mm^2 were applied. Wang *et al*[30] found an increase in the tumor blood flow induced by IV hydralazine injection was accompanied by an increase in the IVIM-derived D* in a mammary adenocarcinoma rat model. In a human brain study, f and D* were correlated separately with the relative blood volume and blood flow derived from dynamic susceptibility contrast enhancement imaging[31]. Joo *et al*[19] reported that IVIM-DWI has the potential to evaluate the early therapeutic effect induced by a vascular disrupting agent named CKD-516 in rabbit VX2 liver tumors. In addition, f and D* also showed a weak negative correlation with the cellular apoptosis of the tumor, indicating that the decreased cellularity also contributed to the changes in f and D*. This may reconfirm the finding that tissue water diffusion also contributes to the observed signal attenuation at low *b*-values. After effective chemotherapy, extracellular spaces would expand, resulting in less restriction of migration of water molecules and weakening the process of pseudo-diffusion. This would then yield a higher D value and lower f and D* than those obtained pre-treatment. Our results and the conclusions from previous studies[13,32] indicated the usefulness of IVIM-DWI for depicting the therapeutic efficiency in gastric cancer before changes in tumor size are evident.

The option of a bi-exponential model for extracting molecular diffusion and microcirculation perfusion information from DWI remains controversial. A previous IVIM study with the *b*-values up to 1500 s/mm^2 found that f increased after treatment, which contradicts our finding[21]. Pang *et al*[23] found that f obtained from *b*-values below 750 s/mm^2 is more in keeping with the increased perfusion in prostate cancer tissue, while f decrease or became indistinguishable from the normal level of prostate tissue when high *b*-values employed. Both tissue microcirculation and water diffusion in the tissue contributed to signal attenuation at a low *b*-value; hence, departure of molecular diffusion at very high *b*-values may influence the perfusion-related parameters[33,34]. Moreover, the IVIM method suffers significantly from the variations in the signal-to-noise ratio and is prone to generate measurement errors when *b*-values are lower than 100 s/mm^2[13]. To date, no consensus has been reached on the magnitude and number of *b*-values that ought to be applied in preclinical and clinical studies. Therefore, more studies are required to optimize the process of image collection and image post-processing for deriving sufficiently accurate parameters based on the original IVIM model.

There are limitations to this study. First, the observation endpoint in this study is too short. Secondly, because of the small sample sizes, serial relative changes in ADC and the IVIM parameters of the tumor were not evaluated in the treatment groups. Therefore, we need further studies with larger sample size and long-term observations to clarity the limitation of this study.

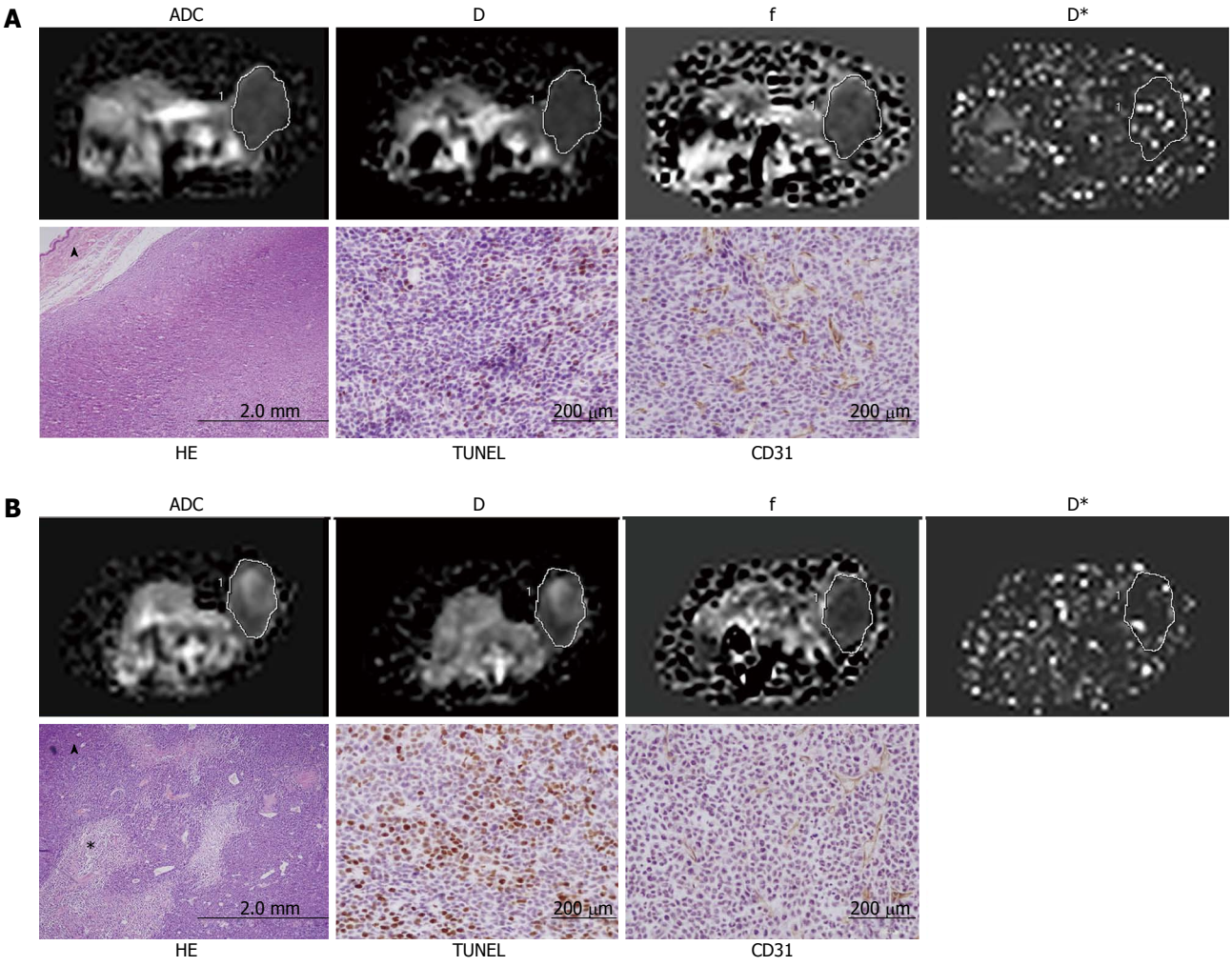


Figure 5 Calculated maps of intravoxel incoherent motion diffusion-weighted imaging parameters and the histopathological images. A: The lower ADC (0.521 × 10^{-3} mm^2/s) and D values (0.475 × 10^{-3} mm^2/s) and the higher f (40.92%) and D* (0.131 mm^2/s) values, which correspond to low necrosis (10%) and cellular apoptosis (7%) and high MVD (36) in the control group; B: Increased ADC (0.875 × 10^{-3} mm^2/s) and D (0.851 × 10^{-3} mm^2/s) values and reduced f (17.20%) and D* (0.098 mm^2/s) values, which correspond to the increased necrosis (18%) and cellular apoptosis (23%) and decreased MVD (17) in the 3-d treatment group. Note high signal intensity within tumor suggesting necrosis. asterisk means necrosis area; The triangle symbol means skin of the mouse. ADC: Apparent diffusion coefficient; MVD: Microvessel density.

In conclusion, IVIM-DWI raises the possibility of an effective, multi-parametric (D, f, D*) imaging method without requirement of gadolinium enhancement. The IVIM method could potentially be used to assess tissue diffusivity changes in addition to evaluate the microcirculatory perfusion of gastric cancer in response to chemotherapy.

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COMMENTS

Background

Chemotherapy is a standard treatment for advanced gastric cancer. Imaging modalities that help to identify early and reliable surrogate markers for the evaluation of chemotherapy response are important in patients with gastric cancer. Intravoxel incoherent motion diffusion-weighted imaging (IVIM-DWI), which allows acquisition of quantitative parameters that reflect tissue diffusivity and tissue microcapillary perfusion, maybe a useful tool that can monitor the early chemotherapy response in terms of gastric cancer tissue diffusion and microvascular perfusion.

Research frontiers

IVIM-DWI has been used to monitor the treatment responses to radiofrequency ablation or a vascular disrupting agent of CKD-516 in rabbit model with VX2 liver tumors, and the response of human locoregionally advanced nasopharyngeal carcinoma to neoadjuvant chemotherapy. DWI is increasingly being applied in the human body, but few studies focused on the gastric lesions due to the limitation of the modality.

Innovations and breakthroughs

In this study, IVIM-DWI with 12 *b*-values less than 800 s/mm^2 was performed to avoid the potential impact of higher *b*-values on the accuracy of the IVIM-derived parameters.

Applications

IVIM-DWI raises the possibility of an effective, multi-parametric (D, f, D*) imaging method that does not require gadolinium enhancement. The IVIM

method could potentially be used to assess tissue diffusivity changes in addition to measuring the microcirculatory perfusion of gastric cancer in response to chemotherapy.

Terminology

DWI is capable of providing a parameter of apparent diffusion coefficient (ADC). The measured ADC, which represents tissue diffusivity, has become a favorite choice in oncologic studies. IVIM-DWI allows acquisition of multi-quantitative parameters that reflect tissue diffusivity and microcirculation perfusion.

Peer-review

The authors have demonstrated the usefulness of IVIM-DWI for monitoring early chemotherapeutic efficacy in a human gastric cancer xenograft model with nude mouse. The manuscript presents interesting and novel findings. The data are well presented and important. However, noted by the authors, the limitation of this study is the short observation endpoint.

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ORIGINAL ARTICLE

Basic Study

MicroRNA-21 promotes phosphatase gene and protein kinase B/phosphatidylinositol 3-kinase expression in colorectal cancer

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Author contributions: Sheng WZ and Chen YS contributed equally to this study, Sheng WZ, Chen YS and Gao WD designed research; Sheng WZ, Chen YS, Gao WD, Tu CT, He J and Zhang B performed research; Gao WD, Tu CT, He J and Zhang B contributed new reagents or analytic tools; Sheng WZ and Chen YS analyzed data; Sheng WZ and Gao WD wrote the paper.

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Abstract

AIM: To explore the regulatory mechanism of the target gene of microRNA-21 (miR-21), phosphatase gene (PTEN), and its downstream proteins, protein kinase B (AKT) and phosphatidylinositol 3-kinase (PI3K), in colorectal cancer (CRC) cells.

METHODS: Quantitative real-time PCR (qRT-PCR) and Western blot were used to detect the expression levels of miR-21 and PTEN in HCT116, HT29, Colo32 and SW480 CRC cell lines. Also, the expression levels of PTEN mRNA and its downstream proteins AKT and PI3K in HCT116 cells after downregulating miR-21 were investigated.

RESULTS: Comparing the miR-21 expression in CRC cells, the expression levels of miR-21 were highest in HCT116 cells, and the expression levels of miR-21 were lowest in SW480 cells. In comparing miR-21 and PTEN expression in CRC cells, we found that the protein expression levels of miR-21 and PTEN were inversely correlated ($P < 0.05$); when miR-21 expression was reduced, mRNA expression levels of PTEN did not significantly change ($P > 0.05$), but the expression levels of its protein significantly increased ($P < 0.05$). In comparing the levels of PTEN protein and downstream AKT and PI3K in HCT116 cells after downregulation of miR-21 expression, the levels of AKT and PI3K protein expression significantly decreased ($P < 0.05$).

CONCLUSION: PTEN is one of the direct target genes

of miR-21. Thus, phosphatase gene and its downstream AKT and PI3K expression levels can be regulated by regulating the expression levels of miR-21, which in turn regulates the development of CRC.

Key words: MicroRNA-21; Protein kinase B; Colorectal cancer; Phosphatidylinositol 3-kinase; Phosphatase and tensin homolog

Core tip: RT-PCR and Western blot were applied to detect the expression level of microRNA-21 (miR-21) and Phosphatase gene (PTEN), including its downstream proteins protein kinase B (AKT) and phosphatidylinositol 3-kinase (PI3K) in colorectal cancer (CRC) cell lines, and to explore the regulatory mechanism of the expression of miR-21 in inhibiting CRC, respectively. Their associations were investigated to clarify whether one of the direct target genes of miR-21 is PTEN. The expression levels of miR-21, PTEN and its downstream proteins AKT and PI3K are regulated and controlled to manage the occurrence and progression of CRC.

Sheng WZ, Chen YS, Tu CT, He J, Zhang B, Gao WD. MicroRNA-21 promotes phosphatase gene and protein kinase B/phosphatidylinositol 3-kinase expression in colorectal cancer. *World J Gastroenterol* 2016; 22(24): 5532-5539 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i24/5532.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i24.5532

INTRODUCTION

MicroRNA (miRNA) is a kind of non-coding macromolecule RNA that includes nearly 22 nucleotides, and it is able to conjugate the 3'UTR of mRNA to facilitate target mRNA degradation and inhibition of the translation process by virtue of lowering gene expression[1-4]. The research of Calin *et al* indicated that approximately 50% of miRNA was located in tumor-related genomic regions. This work also revealed aberrant expression levels in a number of tumors, which was probably on account of the oncogenic and tumor suppressing gene functions of miRNA molecules. In addition, miRNA is involved in the occurrence and progression of human tumors[5-8]. Phosphatase gene (PTEN) is a phosphatase and tensin homologue gene derived from chromosome ten, which is associated with phosphohydrolase; its inactivation induces tumor occurrence in the human body[9-11]. In recent years, the protein kinase B/phosphatase gene/phosphatidylinositol 3-kinase (AKT/PTEN/PI3K) signaling pathway has raised increasing concern, and a number of studies found that the atypical AKT/PTEN/PI3K signaling pathway was intimately linked with numerous tumor occurrences and progression, immunity, drug resistance, metastasis, angiogenesis, *etc.*[12-16]. In addition, the AKT/PTEN/PI3K signaling pathway has been reported in pulmonary, nasopharyngeal, gastric and renal tumors, as well as in neuroglioma[17-21]. However, literature with regard to colorectal cancer (CRC) is rare. Moreover, there were reports that revealed that microRNA-21 (miR-21) could regulate the AKT/PTEN/PI3K signaling pathway to promote tumor occurrence and progression, and even tumor invasion[22-25]. Hence, quantitative real-time PCR (qRT-PCR) and Western blot were applied to detect the expression level of miR-21 and PTEN, including its downstream proteins AKT and PI3K, in CRC cell lines. Furthermore, the mechanism of miR-21 expression in inhibiting CRC and their correlations were also explored. This study will provide a theoretical and experimental basis for the early diagnosis and therapy of CRC.

MATERIALS AND METHODS

Experimental materials

Primary reagents and equipment: PTEN antibody, PI3K mouse anti-human monoclonal antibody, immunohistochemistry (IHC) kit and AKT rabbit anti-human polyclonal antibody were purchased from Beijing Zhongshan Golden Bridge Biotechnology Company. Quantitative RT-PCR kit and miR-21 primer were purchased from Takara. Fluorescent quantitative PCR detection system was purchased from ABI (United States). The PCR instrument was purchased from Bio-Rad (United States). The inverted microscope was purchased from Olympus (Japan). The refrigerated centrifuge was purchased from Thermo Scientific (United States). HCT116, HT29, Colo32 and SW480 CRC cell strains were all purchased from the Cell Bank of the Chinese Academy of Sciences.

Preparation of primary reagents: Requisite reagents: phosphate buffer solution (PBS), bovine serum albumin (BSA) solution, Tris-buffered saline and Tween-20 (TBST) buffer, BSA blocking buffer, 0.25% trypsin solution, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis buffer, transmembrane buffer, SDS-PAGE separation and stacking gel.

Experimental methods

CRC cell culture and transfection: A Dulbecco's modified Eagle's medium (DMEM) high glucose medium with 10% calf serum was used to culture HCT116, Ht29, Colo32 and SW480 CRC cell strains in 5% CO_2 at 37 ℃. Cells in the logarithmic phase were used in experiments. All cells were sorted into three groups: miR-21 inhibition group (IG), negative control (NC) and blank control (BC). HCT116 cells were inoculated in 500 μL of medium (no antibiotics) to 30%-60% degree to spare. Then, 20 pmol/L of miR-21 inhibitor was

diluted with 50 mL of DMEM medium (no serum) and incubated for five minutes at room temperature after mixing. Next, 1 μL of mixing Lipofectamine 2000 was diluted in 50 μL of DMEM (no antibiotics and serum) and incubated at room temperature for 5 min. The diluted miR-21 inhibitor was mixed with Lipofectamine 2000 and incubated at room temperature for 20 min. Then, a 100-μL transfection buffer was added, mixed and incubated for six hours in an incubator (5% CO_2 at 37 ℃). Afterward, the medium was changed to normal medium and cells were incubated for another 48-72 hours for detection.

Transwell assay: Pre-cooling non-serum DMEM was used to dilute the Matrigel matrix gel. Then, it was paved on the chamber of the polycarbonate filtering membrane followed by inoculation of diluted HCT116 cells (100 μL). Afterwards, 10% fetal calf serum was added to the lower chamber. After 24 h of culture in 5% CO_2 at 37 ℃, the chamber was washed twice, stained with 0.1% crystal violet for five minutes, and washed again. Cells were randomly counted in five views at 100 × objective and the average was calculated. This was performed in three replicates for each group.

Real-time quantitative RT-PCR to detect miR-21 and PTEN mRNA levels: Total RNA was extracted using Trizol RNA extraction methods, based on the Molecular Clone Technique Experimental Manual. One mL of Trizol reagent could be added to approximately 100-mg tissues. A mortar was used to grind the tissues to powder, which eventually reached complete decomposition. The lysate was drawn into a 1.5-mL tube and incubated for 10 min on ice. After centrifuging at 12000 rpm for 10 min at 4 ℃, the supernatant was drawn into a new 1.5-mL tube. Isopropanol in equal volume was added and mixed, plated on ice for 10 min, and centrifuged at 12000 rpm for another 10 min at 4 ℃. The supernatant was discarded, 1 mL of 75% ethanol was added, the precipitate was washed, and centrifuged for another five minutes. Afterwards, the supernatant was discarded, placed into a tube in room temperature for 10 min, waited until the residue entirely volatilized, then 100 μL of diethyl pyrocarbonate H_2O was added to dissolve the precipitate, and it was stored in liquid nitrogen for use. Extracted RNA purity and concentration was detected using a spectrophotometer. The normal value of optical density (OD) OD_{260}/OD_{280} was between 1.8 and 2.1, respectively. Then, cDNA was synthesized by reverse transcription reaction. RT-PCR was used to detect miR-21 reaction conditions: 95 ℃ for 10 s, one cycle, 95 ℃ for 5 s, 60 ℃ for 34 s, 45 cycles; computational formula: relative amount = $2^{-\Delta\Delta CT}$, where -ΔΔCT = (CTmiR-21-CTu6) tumor-(CTmiR-21-CTu6) normal tissue. RT-PCR was used to detect PTEN mRNA reaction conditions: 95 ℃ for 10 s, one cycle, 95 ℃ for 5 s, 60 ℃ for 20 s, 45 cycle; and PTEN mRNA expression level was calculated on the basis of ΔCt = Ct(PTEN) - Ct(β-actin), where Folds = $2^{-\Delta\Delta CT}$, and the average result was used with three repeats.

Western blot: After 48-72 h of transfection, the medium was discarded and 100 μL of RIPA lysate was added into each well. The lysate was mixed for 5 min, centrifuged at 12000 rpm for 15 min at 4 ℃, and the supernatant was stored for use. Samples mixed with 5 × loading were boiled for 10 min at 95 ℃ and loaded after centrifugation. Eight μL of purified and desalted antibody was loaded, and 2-3 μL of the marker was loaded. The antibody was confirmed according to the marker position. Electrophoresis: stacking gel in 80 V for 20-30 min, and separation gel in 120 V for 40 min. Transmembrane: 300 mA for 90 min. After transmembrane, the membrane was blocked with non-fat milk, washed with PBS for three times, and incubated with the primary antibody (PTEN antibody, PI3K antibody, AKT antibody and β-actin antibody; 1:500 dilution) overnight at 4 ℃. On the next day, cells were washed with TBST for seven times and incubated with the secondary antibody (horseradish peroxidase-labeled goat-anti rabbit secondary antibody, 1:2500 dilution) for one hour. Then, cells were washed with TBST for another six times. Chemiluminescence reagent A and B solutions were mixed and added on the membrane based on a 1:1 proportion, and β-actin protein was used as an internal reference.

Statistical analysis

SPSS 19.0 was used to analyze all data; measurement data were presented as mean ± SD. The *t*-test was used to compare miR-21 and PTEN expression levels, and the variance analysis method was used to compare the mean value of transfection in the IG, NC and BC groups. $P < 0.05$ was considered statistically significant.

RESULTS

miR-21 and PTEN expression in CRC cells

miR-21 had the highest protein expression in HCT116 cells, but had the lowest expression in SW480 cells (Figure 1). In contrast, PTEN protein expression was the lowest in HCT116 cells, but had the highest expression in SW480 cells (Figure 2). The protein expression level between miR-21 and PTEN was inversely related, and the difference was statistically significant ($P < 0.01$).

Invasive ability changes in cells after transfection

In counting the cells that crossed after transfection in the Transwell assay, we found that cells transfected with miR-21 in IG were reduced by 39.1% compared to BC and 36.9% compared to NC. However, the difference between BC and NC was not statistically significant (Figure 3).

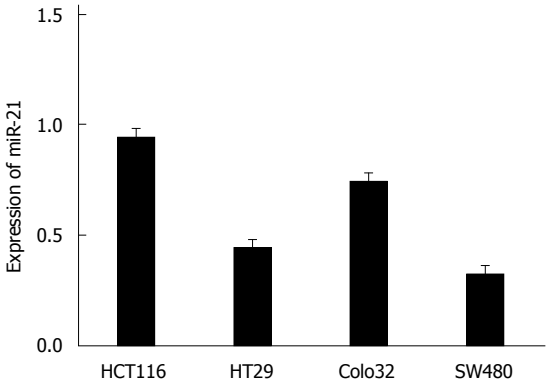

Figure 1 Expression of miR-21 in colorectal cancer cell lines (*n* = 3).

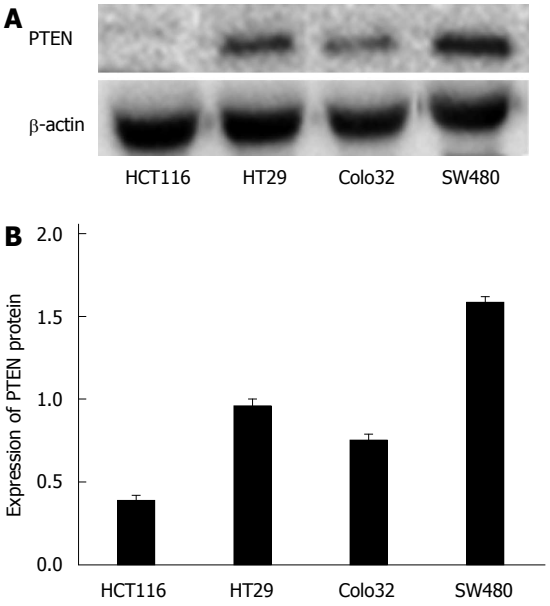

Figure 2 Expression of PTEN protein in colorectal cancer cell lines. A: Western blot assay; B: the expression of PTEN in colorectal cancer cell lines (*n* = 3).

RT-PCR detection of miR-21 expression after transfection

miR-21 expression levels after transfection in IG, NC and BC are shown in Figure 4. It can be observed that miR-21 expression levels in BC and NC were higher than in IG, and the difference was statistically significant ($P < 0.05$).

PTEN mRNA and protein expression in HCT116 cells after downregulation of miR-21

As shown in Figure 5A, after reducing the expression of miR-21, PTEN mRNA expression levels in HCT116 cells revealed no statistical significance after transfection in IG, NC and BC ($P > 0.05$). As illustrated in Figure 5B, when miR-21 was downregulated, PTEN protein expression levels were markedly elevated, and the difference was statistically significant ($P < 0.05$). The difference between BC and NC was not statistically significant ($P > 0.05$)

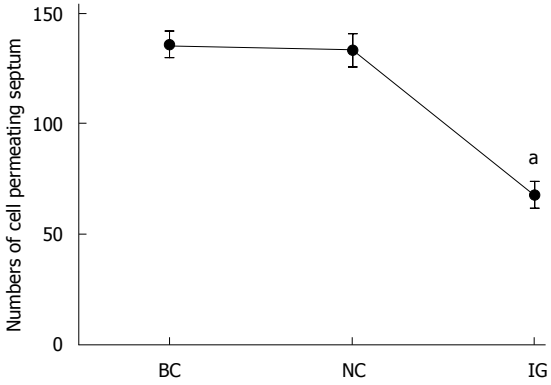

Figure 3 Changes in cell invasion ability after transfection ($^aP < 0.05$, $n = 3$). IG: Inhibition group; NC: Negative control; BC: Blank control.

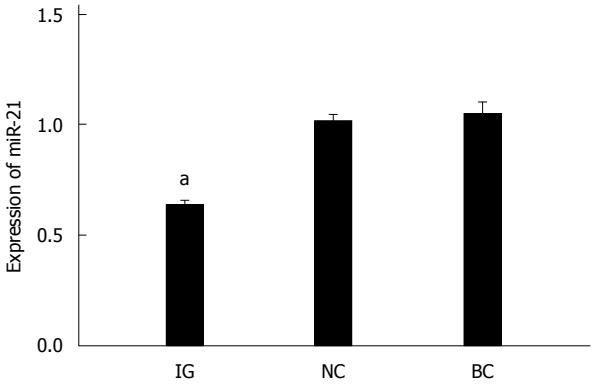

Figure 4 Effect of the miR-21 inhibitor on miR-21 expression in HCT116 cells ($^aP < 0.05$, $n = 3$). IG: Inhibition group; NC: Negative control; BC: Blank control.

PTEN and its downstream proteins AKT and PI3K expression in HCT116 cells after downregulating miR-21

As shown in Figure 6, PTEN protein expression levels in HCT116 cells were elevated in IG, and the difference was statistically significant ($P < 0.05$). However, AKT and PI3K expression levels in HCT116 cells in IG decreased; and the difference was statistically significant ($P < 0.05$).

DISCUSSION

Much research has indicated that CRC occurrence in patients is a complicated process, which consists of multiple stages and factors. One momentous feature is its oncogenic activation and tumor-suppressing gene expression dysregulation or absence[26,27]. In recent years, it has been found miRNA possesses oncogenic and tumor suppressing gene functions[22]. One representative miRNA, miR-21, possesses a crucial oncogenic function and exhibits high expression levels in a number of tumors; it is closely associated with tumor occurrence and chemotherapy sensitivity[28,29]. PTEN is derived from chromosome ten, and acts as a tumor-suppressing gene. PTEN exerts vital effects on cell growth, proliferation, migration, signal transmission,

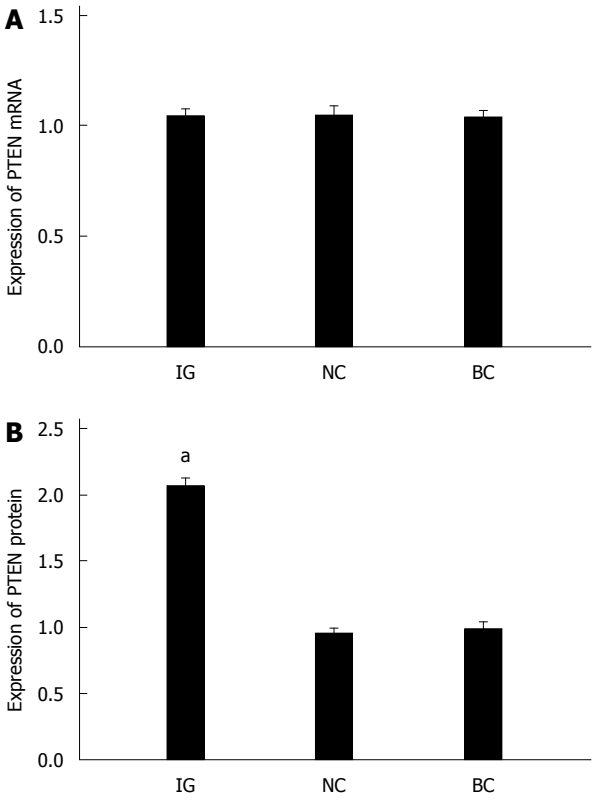

Figure 5 Effect of the miR-21 inhibitor on PTEN mRNA (A) and protein (B) expression in HCT116 cells ($^aP < 0.05$, $n = 3$). IG: Inhibition group; NC: Negative control; BC: Blank control.

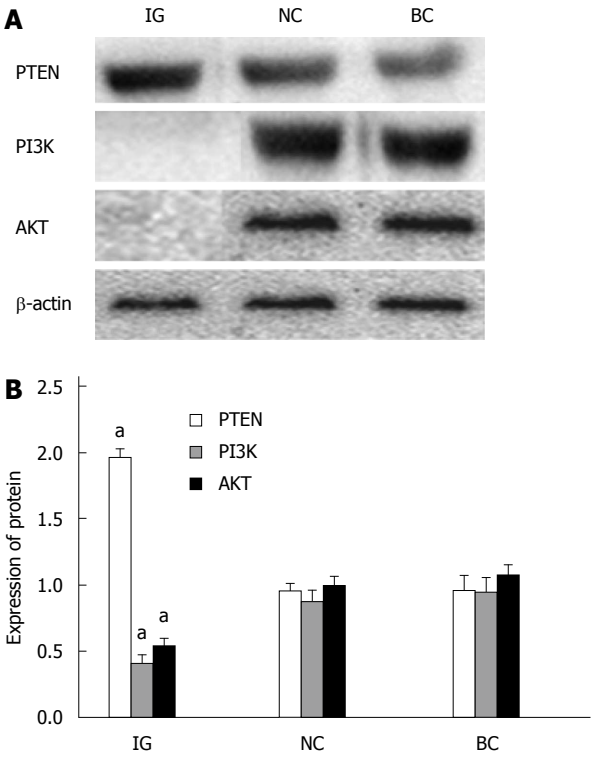

Figure 6 Protein expression of PTEN/AKT/PI3K in colorectal cancer HCT116 cell lines. A: Western blot assay; B: Protein expression in HCT116 cell lines ($^aP < 0.05$, $n = 3$). IG: Inhibition group; NC: Negative control; BC: Blank control.

invasion and apoptosis; it has an inhibitory role on tumor cell growth, invasion, proliferation, metastasis and apoptosis[30]. The AKT/PTEN/PI3K signaling pathway plays a significant part in regulating cell growth, metabolism, differentiation and apoptosis; it is closely associated with tumor occurrence and progression[31-36]. PTEN protein phosphatase activity (dephosphorylation) can inhibit AKT function, leading to cell apoptosis. Furthermore, the deletion or mutation of PTEN is able to enhance AKT activity to increase PI3K expression[37]. AKT/PTEN/PI3K signaling pathway activation is capable of inhibiting cell apoptosis to facilitate cell differentiation and proliferation. This also exerts a crucial role in tumor occurrence and progression, and participates in tumor metastasis and invasion[38-44].

miR-21 and PTEN expression in CRC cells

miR-21 and PTEN protein expression was detected in HCT116, HT29, Colo32 and SW480 CRC cell lines through RT-PCR and Western blot. Results revealed that miR-21 expression level was highest in HCT116 cells and lowest in SW480 cells. Furthermore, PTEN expression level was lowest in HCT116 cells and highest in SW480 cells. The protein expression level between miR-21 and PTEN was inversely correlated. These results indicate that miR-21 could be involved in the regulation of PTEN protein expression. In addition, due to the high expression level of miR-21 in HCT116 cells, HCT116 cells were selected for this study. Transwell invasion assay revealed that the invasive capacity of tumor cells was obviously reduced after inhibiting CRC cell miR-21 expression, which in turn indicates that miR-21 probably plays a promoting role in cell malignancy transformation.

miR-21 and PTEN mRNA and protein expression in HCT116 cells after downregulating miR-21

After downregulating the expression of miR-21, the expression level of miR-21 in HCT116 cells in IG was obviously lower compared to NC and BC, which illustrates the successful inhibition of miR-21. As a known miR-21 target gene, PTEN has been verified to be a target gene of miR-21 by Western blot. When we inhibited the expression of miR-21, PTEN mRNA expression levels did not reveal apparent change, but protein levels were obviously elevated; which indicates that miR-21 could regulate PTEN expression at the post-transcriptional level and that PTEN was a target gene for miR-21. Meng *et al*[45] recently found that miR-21 targeted tumor-suppressing gene PTEN in liver and bile duct cancer cells to promote tumor growth, invasion and migration, manifesting that PTEN was a target gene of miR-21.

PTEN and its downstream AKT and PI3K expression in HCT116 cells after reducing miR-21 expression

After inhibiting the expression of miR-21, PTEN protein

level was significantly elevated, but its downstream AKT and PI3K expression obviously decreased, indicating that miR-21 could regulate and control PTEN and the expression level of downstream AKT and PI3K in terms of its effects on tumor cell invasion and migration. Certain studies have shown that change in miR-21 expression level is able to induce PTEN downstream molecule AKT phosphorylation, metalloproteinase 2 and focal adhesion kinase expression; these are altered to restrain tumor cell invasion and migration[45-50].

In conclusion, our study revealed that PTEN is one of the direct target genes of miR-21. By altering miR-21 expression, downstream AKT and PI3K expression levels could be regulated and controlled, in order to control CRC occurrence and progression. Nevertheless, the mechanisms of miR-21 and PTEN function, as well as their effects in tumors, need to be further investigated. As more in-depth research on miRNA is being undertaken, especially regarding tumor cell regulatory mechanisms, more roles and targets of miR-21 will probably be unraveled.

COMMENTS

Background

Colorectal cancer (CRC) is complicated and combines multiple stages and factors. In recent years, we have found that miRNA possesses oncogenic and tumor-suppressing gene functions. One representative miRNA, microRNA-21 (miR-21), possesses a crucial oncogenic function that shows high expression levels in a number of tumors; this is closely associated with tumor occurrence and chemotherapy sensitivity. PTEN acts as a tumor-suppressing gene derived from chromosome ten. PTEN exerts vital effects in cell growth, proliferation, migration, signal transmission, invasion and apoptosis; it has an inhibitory role in tumor cell growth, invasion, proliferation, metastasis and apoptosis. The AKT/PTEN/PI3K signaling pathway plays a significant part in regulating cell growth, metabolism, differentiation, and apoptosis; it is closely associated with tumor occurrence and progression.

Research frontiers

In addition, the AKT/PTEN/PI3K signaling pathway has been reported in pulmonary, nasopharyngeal, gastric and renal tumors, as well as in neuroglioma; however, literature with regard to CRC is rare. Moreover, there have been reports that revealed that miR-21 could regulate the AKT/PTEN/PI3K signaling pathway, in order to promote tumor occurrence and progression, and even tumor invasion. Hence, RT-PCR and Western blot were applied to detect the expression levels of miR-21 and PTEN, including its downstream proteins AKT and PI3K in CRC cell lines, as well as the inhibition of miR-21 expression in CRC cell lines. Furthermore, their association was also explored. This study hopes to provide a theoretical and experimental basis for the early diagnosis and therapy of CRC.

Innovations and breakthroughs

RT-PCR and Western blot were applied to detect the expression level of miR-21 and PTEN including its downstream proteins AKT and PI3K, and the inhibition of miR-21 expression in CRC cell lines, respectively. This association was also investigated to demonstrate that PTEN was one of the direct target genes of miR-21. Through regulating and controlling the expression level of miR-21, PTEN and its downstream proteins AKT and PI3K expression level are regulated to control the occurrence and progression of CRC.

Applications

This study revealed that PTEN was one of the direct target genes of miR-21, and that its downstream AKT and PI3K expression levels could be regulated and controlled through altering miR-21 expression, in order to control CRC occurrence and progression. Nevertheless, the mechanisms of miR-21 and PTEN function, as well as their effects in tumors, need to be further investigated. As in-depth research on mRNA is currently being implemented, particularly regarding the regulatory mechanism of tumor cells, more roles and targets of miR-21 will probably be unraveled.

Peer-review

This is an interesting manuscript about the miR-21 promotion of PTEN and its downstream proteins AKT and PI3K in CRC. Over all, this study is well designed and the manuscript is well written.

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ORIGINAL ARTICLE

Basic Study

Effects of sphincter of Oddi motility on the formation of cholesterol gallstones

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Author contributions: Rong ZH and Zhang ZH contributed equally to this work; Zhang ZH, Rong ZH, Xian GZ, and Qin CK designed the research; Rong ZH, Chen HY, Wang XX, Wang ZY, and Ma BZ performed the research; Rong ZH and Zhang ZH analyzed the data; Rong ZH and Zhang ZH wrote the paper.

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Institutional review board statement: The study was reviewed and approved by the Provincial Hospital Affiliated to Shangdong University Institutional Review Board.

Institutional animal care and use committee statement: The animal protocol was designed to minimize pain or discomfort to the animals. The animals were acclimatized to laboratory conditions (room temperature 23 ℃, 12-h light and dark cycle, 50% humidity, ad libitum access to food and water) for two weeks prior to experimentation. All animals were euthanized by barbiturate overdose (intravenous injection, 150 mg/kg pentobarbital sodium) for tissue collection.

Conflict-of-interest statement: The authors declare there is no conflict of interest related to this study.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at zhangzhenhai410@126.com. Participants gave informed consent for data sharing.

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Abstract

AIM: To investigate the mechanisms and effects of sphincter of Oddi (SO) motility on cholesterol gallbladder stone formation in guinea pigs.

METHODS: Thirty-four adult male Hartley guinea pigs were divided randomly into two groups, the control group (n = 10) and the cholesterol gallstone group (n = 24), which was sequentially divided into four subgroups with six guinea pigs each according to time of sacrifice. The guinea pigs in the cholesterol gallstone group were fed a cholesterol lithogenic diet and sacrificed after 3, 6, 9, and 12 wk. SO manometry and recording of myoelectric activity were obtained by a multifunctional physiograph at each stage. Cholecystokinin-A receptor (CCKAR) expression levels in SO smooth muscle were detected by quantitative real-time PCR (qRT-PCR) and serum vasoactive intestinal peptide (VIP), gastrin, and cholecystokinin octapeptide (CCK-8) were detected by enzyme-linked immunosorbent assay at each stage in the process of cholesterol gallstone formation.

RESULTS: The gallstone formation rate was 0%, 0%, 16.7%, and 83.3% in the 3, 6, 9, and 12 wk groups, respectively. The frequency of myoelectric activity in the 9 wk group, the amplitude of myoelectric activity in the 9 and 12 wk groups, and the amplitude and the frequency of SO in the 9 wk group were all significantly decreased compared to the control group. The SO basal pressure and common bile duct pressure increased markedly in the 12 wk group, and the CCKAR expression levels increased in the 6 and 12 wk groups compared to the control group. Serum VIP was elevated significantly in the 9 and 12 wk groups and gastrin decreased significantly in the 3 and 9 wk groups. There was no difference in serum CCK-8 between the groups.

CONCLUSION: A cholesterol gallstone-causing diet can induce SO dysfunction. The increasing tension of the SO along with its decreasing activity may play an important role in cholesterol gallstone formation. Expression changes of CCKAR in SO smooth muscle and serum VIP and CCK-8 may be important causes of SO dysfunction.

Key words: Cholesterol gallstone; Sphincter of Oddi; Manometry; Myoelectric activity; Cholecystokinin-A receptor

Core tip: This study investigated the role of sphincter of Oddi (SO) motility in cholesterol gallstone formation in a guinea pig model. The myoelectric activity and manometry of SO were measured at different stages of stone formation. As SO motility is controlled by neurological and hormonal factors, we detected the expression of serum vasoactive intestinal peptide (VIP), gastrin, cholecystokinin octapeptide (CCK-8), and CCK-A receptor (CCKAR) in the SO at different stages of stone formation. We found that a cholesterol gallstone-causing diet can induce SO dysfunction and expression changes of CCKAR in SO smooth muscle and serum VIP and CCK-8 may be important causes of SO dysfunction.

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INTRODUCTION

Gallstone disease is one of the most common digestive disorders requiring hospital admission in Western countries, with a morbidity of 10%-15% in adults[1]. In China, the incidence of cholesterol gallstones has been increasing in the past few decades due to changing lifestyles[2]. Cholesterol gallstone formation is a complicated process and is still not fully understood. Cholesterol supersaturation of bile, biliary stasis, and mucus hypersecretion are universally known to be important factors in the process of cholesterol gallstone formation[3,4]. Among them, biliary stasis is thought to be a key factor because it allows time for cholesterol nucleation and then retention of the precipitated microcrystals[4,5]. The sphincter of Oddi (SO) may play an important role in gallstone formation because it is the only way by which bile is discharged into the duodenum. There are very few reports investigating the relationship between SO motility and cholesterol gallstone formation, and the conclusions are controversial[6-8].

It is well-established that cholecystokinin (CCK) is one of the major gastrointestinal hormones responsible for gallbladder contraction and SO relaxation[9]. The biological actions of CCK in the alimentary tract are mediated by the CCK-A receptor (CCK-AR)[10]. A series of studies focused on the expression level of CCK-AR in the gallbladder in the pathogenesis of cholesterol gallstone disease[5,11]. However, no report on CCK-AR expression in the SO in animals with gallstones is available yet.

The aim of this study was to investigate the role of SO motility in cholesterol gallstone formation in a guinea pig model. The myoelectric activity and manometry of SO were measured at different stages of stone formation in this model. As SO motility is controlled by neurological and hormonal factors, we detected serum vasoactive intestinal peptide (VIP), gastrin, CCK-8, and CCK-AR expression in the SO at different stages of stone formation.

MATERIALS AND METHODS

Experimental animals

Thirty-four adult male Hartley guinea pigs, weighing between 230 g and 270 g, were purchased from Huishan Jiangnan Laboratory Animal Company (License SCXK SU: 2009-0005). The animals were housed in climate-controlled rooms under an alternating 12-h light and dark cycle and permitted continuous access to food and water. After a 2-wk equilibration, the animals were divided randomly into two groups (control and cholesterol stone groups): the control group (n = 10) was fed a normal diet, and the cholesterol stone group (n = 24) was sequentially divided into four subgroups with six guinea pigs each according to time of sacrifice, fed a cholesterol lithogenic diet[6], and sacrificed after 3, 6, 9, and 12 wk. The lithogenic diet consisted of 1% cholesterol[10] (purchased from Trophic Animal Feed High-Tech Co. Ltd., Nantong, China). Gallstones were defined as macroscopically visible sediment. The calculi were tested by infrared spectrometry to verify the sample as cholesterol gallstones. SO manometry and myoelectric activity of the guinea pigs were determined at 3, 6, 9, and 12 wk.

Table 1 Primer sequences of *CCKAR* and *GAPDH*

Genes	Forward primer (5′-3′)	Reverse primer (5′-3′)
CCKAR	ACGGAGGGTAGTGAACTCCA	TCGCAGGCAGAAGTGATGTT
GAPDH	GCACCGTCAAGGCTGAGAAT	CATCACGAACATAGGGGCATC

CCKAR: Cholecystokinin-A receptor; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

Measurement of myoelectric activity of the SO

At the end of the feeding period, the animals were anesthetized by injecting pentobarbital sodium (45 mg/kg) into the peritoneal cavity. The guinea pigs were fixed in the supine position, and the skin of the superior abdomen was prepared and sterilized. A longitudinal incision was made and the papilla determined. Details of the myoelectric system were described previously[12]. In brief, two polar hook metal electrodes were inserted 0.2 mm into the subserosa of the SO by megaloscope (× 10 magnification). The fan-out of the two signals was connected with the two polars of the physiological recorder (BL-420 F; Chengdu Taimeng Software, Chengdu, China), and a piece of metal needle was inserted into the legs of the animals to connect with the earth pole of the recorder. The myoelectric signal was collected by the electrode, imported into the computer, and stored after processing by a physiological recorder and the software system specialized in electromyographic signals. The setup parameters were as follows: scanning speed, 500 ms/div; sensitivity, 200 μV; time parameter, 1 s; and frequency filtering, 10 Hz. Finally, the myoelectric figure was dealt with by digital filtering of 10-30 Hz.

SO manometry

Details of manometry were described previously[12]. In brief, a manometry catheter was modified from a pedo bi-lumen central venous catheter (4F and 30 cm long). The catheter was inserted into the common bile duct (CBD) and SO through the duodenal ampulla. The pressure transducer was used for receiving the dynamic pressure change from the manometric lumen. The frequency of SO phasic contraction, SO amplitude, SO basal pressure, and CBD pressure were measured and recorded. A physiological recorder and relevant manometry program were used to record and analyze the tracings.

Detection of serum VIP, gastrin, and CCK-8

Four milliliters of venous blood were obtained from the guinea pigs in the early morning before they were sacrificed and placed in a test tube. The blood was centrifuged at 1500 r/min for 15 min, and serum was isolated, placed in Eppendorf tubes and stored at -70 ℃. Serum VIP, gastrin, and CCK-8 were measured by enzyme-linked immunosorbent assay (ELISA). The ELISA testing kit was supplied by USCN Life Science Inc. (Houston, TX, United States).

Quantification of CCKAR mRNA in SO tissues

The SO of each animal was quickly removed and transferred to normal saline after animals were euthanized. The smooth muscle was removed carefully using sharp dissection. Total RNA from SO tissue samples was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, United States). Every 50 mg of SO tissue sample was extracted with 1 mL of TRIzol reagent according to the manufacturer's protocol. One microgram of total RNA was used for the synthesis of cDNA using a FastQuant RT Kit and target genes were assayed using SYBR Green Real-time PCR Master Mix (*via* Roche Light Cycler, Roche, Basel, Switzerland) with their respective primers. The PCR conditions were as follows: 95 ℃ for 30 s; 40 cycles of 95 ℃ for 5 s, 57 ℃ for 10 s, and 72 ℃ for 15 s. Transcription levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as an internal control to calculate fold induction, and the fold changes in transcription levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Primer sequences of CCKAR and GAPDH are shown in Table 1.

Statistical analysis

Statistical analysis was performed using Student's *t* test. Data were analyzed with software SPSS 17.0 (SPSS Inc. Chicago, IL, United States), and $P < 0.05$ was set as the level of significance. The results are expressed as mean ± SE.

RESULTS

Gallstone formation rate

No gallstone was found in guinea pigs in the control group. In contrast, the gallstone formation rate in the cholesterol stone group fed with a cholesterol lithogenic diet was 0%, 0%, 16.7%, and 83.3% in the 3, 6, 9, and 12 wk groups, respectively.

SO myoelectric activity analysis

Compared with the control group, the frequency of myoelectric activity decreased markedly in the 9 wk group (Figure 1A and B, Table 2). The amplitude of myoelectric activity decreased significantly in the 9 and 12 wk groups (Figure 1A-C, Table 2).

SO manometry analysis

Compared with the control group, the amplitude and frequency of SO decreased significantly in the 9 wk group (Table 3), and SO basal pressure and common

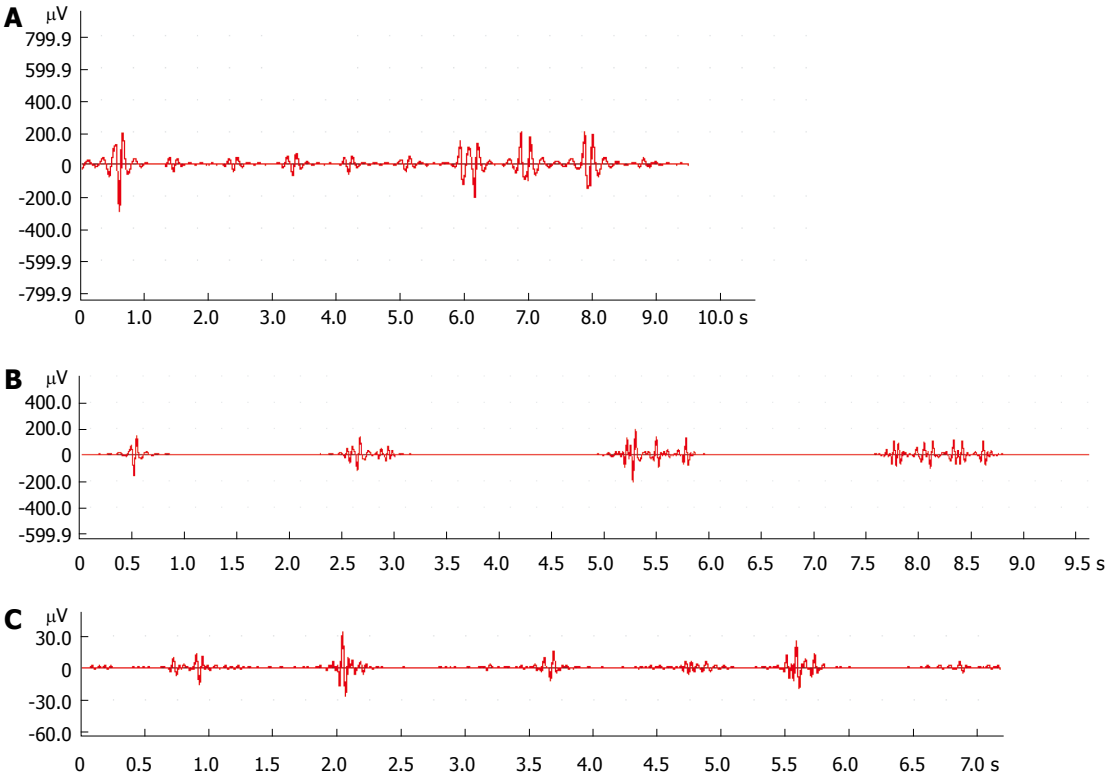

Figure 1 Sphincter of Oddi myoelectric activity analysis. A: The frequency and amplitude of myoelectric activity in the control group; B: The frequency and amplitude of myoelectric activity in the 9 wk group; C: The frequency and amplitude of myoelectric activity in the 12 wk group.

Table 2 Changes of sphincter of Oddi myoelectric activity in the process of gallstone formation

Group	Amplitude	Frequency
Control group	142.45 ± 71.25	16.56 ± 4.05
3-wk group	125.06 ± 59.76	13.70 ± 2.88
6-wk group	152.96 ± 81.05	15.10 ± 4.13
9-wk group	100.27 ± 50.42[a]	8.68 ± 2.35[a]
12-wk group	40.60 ± 15.03[c]	13.80 ± 3.79

[a]$P < 0.05$, [c]$P < 0.01$ *vs* the control group.

bile duct pressure increased significantly in the 12 wk group (Figure 2, Table 3).

Changes in serum VIP, gastrin and CCK-8

Compared with the control group, serum VIP was elevated significantly in the 9 and 12 wk groups (Table 4), and serum gastrin was decreased significantly in the 3 and 9 wk groups (Table 4). There was no difference in serum CCK-8 between the groups (Table 4).

Quantitative expression of CCK-AR mRNA

Compared with the control group, expression levels of the CCK-A receptor mRNA were increased significantly in the 6 and 12 wk groups (Figure 3).

DISCUSSION

Gallstone disease is one of the most common and most expensive digestive disorders requiring admission to the hospital[13-15], with a prevalence of 10%-15% in adults in Europe and the United States. In Western countries, cholesterol gallstones account for 80%-90% of gallstones at cholecystectomy[15], but the mechanism underlying the pathogenesis of cholesterol gallstone disease is not completely understood.

Epidemiological evidence indicates that multiple environmental factors and genetic elements are involved in cholesterol gallstone formation. Among them, biliary stasis is thought to be an important factor. A series of studies in human and animal models have shown that formation of cholesterol gallstones is causatively related to decreased gallbladder contractility[16]. Since SO is the only gate through which bile is discharged into the duodenum, bile filling and excretion of the gallbladder are closely related to the motility state of the SO[17]. However, there is limited research on the role of SO motility in the process of cholesterol gallstone formation. SO manometry (SOM) is the only method that can assess directly the motor function of the SO and is considered the gold standard for assessing SO dysfunction (SOD)[18]. However, manometry changes of the SO in cholelithiasis are controversial. Research by Pang *et al*[19] indicated that the base pressure of the SO was significantly increased in rabbits fed a cholesterol lithogenic diet. On the other hand, a study in prairie dogs showed that SO resistance remained normal throughout the period of gallstone formation. These completely different results may be species dependent. Meanwhile, the mechanical activity recorded may not represent the features of

Table 3 Sphincter of Oddi manometry in the process of gallstone formation

Group	SO basal pressure	CBD pressure	Amplitude of SO	Frequency of SO
Control group	26.59 ± 8.16	23.25 ± 8.35	8.72 ± 2.05	11.27 ± 3.74
3-wk group	21.25 ± 1.38	18.48 ± 1.94	8.33 ± 3.85	11.50 ± 1.64
6-wk group	27.57 ± 8.67	25.82 ± 8.26	8.03 ± 3.15	9.33 ± 3.27
9-wk group	34.11 ± 11.56	32.15 ± 11.64	5.89 ± 1.41[a]	7.67 ± 3.44[a]
12-wk group	52.38 ± 12.84[c]	50.11 ± 12.59[e]	6.82 ± 1.34	10.60 ± 3.51

[a]$P < 0.05$, [c]$P < 0.01$, [e]$P < 0.001$ *vs* the control group. SO: Sphincter of Oddi; CBD: Common bile duct.

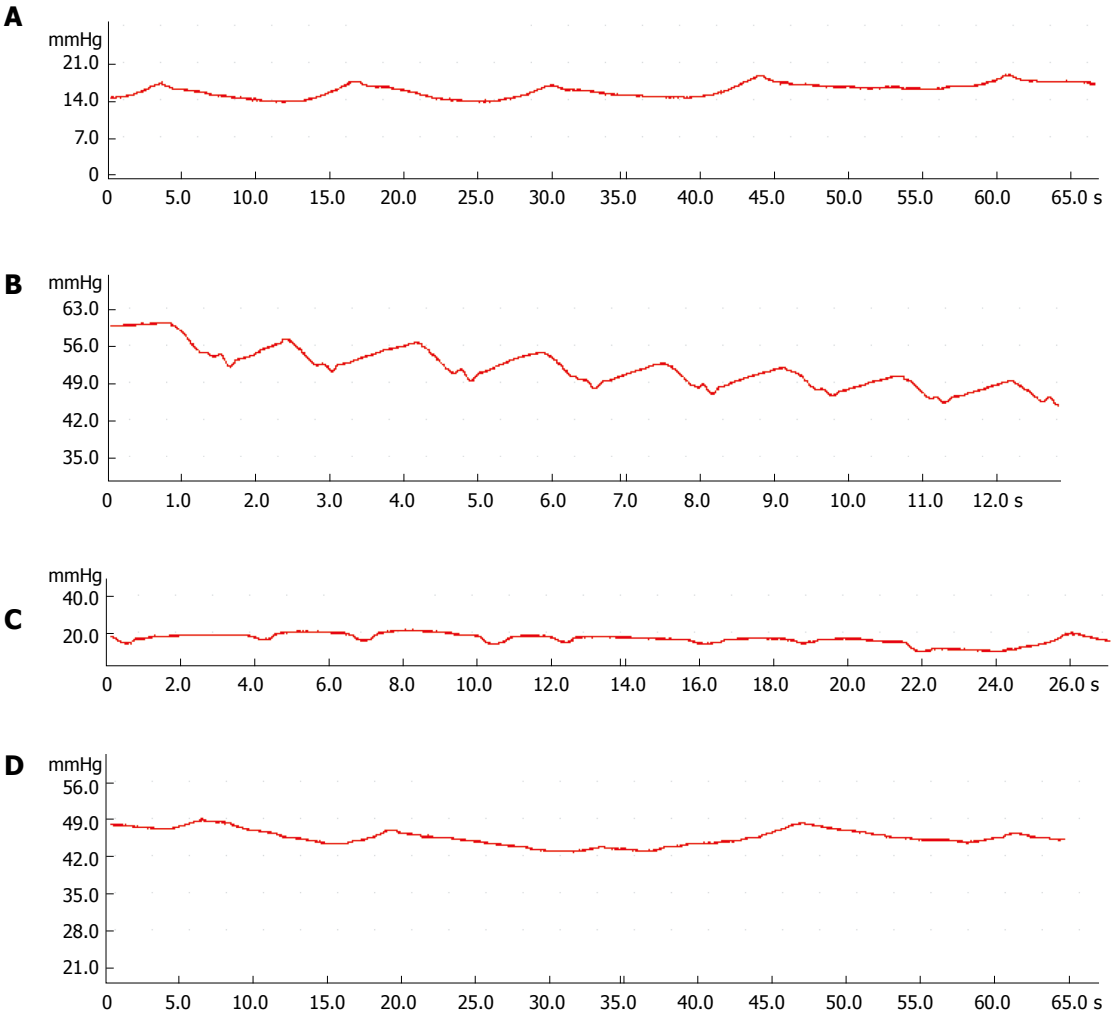

Figure 2 Sphincter of Oddi manometry analysis. A: Sphincter of Oddi (SO) basal pressure in the control group; B: SO basal pressure in the 12 wk group; C: Common bile duct pressure in the control group; D: Common bile duct pressure in the 12 wk group.

SO activity, especially in the relaxed SO. Another measurement reflecting SO function is recording the myoelectric activity of the SO[20].

In this study, we investigated whether SOD was present and what role it played in the process of cholesterol gallstone formation in guinea pigs. SOM and myoelectric activity of SO were investigated simultaneously at different stages of stone formation. We found that gallstones did not occur until 9 wk, with an incidence rate of 16.7%, increasing to 83.3% after 12 wk. SO myoelectric activity analysis indicated that the frequency and amplitude of myoelectric activity decreased significantly in the 9 wk group compared to control. Subsequent SO manometry analyses showed the same result: both SO amplitude and SO frequency decreased significantly in the 9 wk group, and the most important indications, the SO basal pressure and common bile duct pressure, increased significantly in the 12 wk group. All these findings are consistent with weakening of the myoelectric activity of SO and gradual increasing of SO tension in the process of gallstone formation. Disturbance of SO motor function impedes the flow of bile into the duodenum, and biliary stasis occurs.

Table 4 Changes in VIP, gastrin and CCK-8 in the process of cholesterol stone formation (pg/mL)

Group	3-wk	6-wk	9-wk	12-wk
VIP				
Control	9.36 ± 2.72	9.30 ± 8.91	6.91 ± 3.14	8.39 ± 0.99
Cholesterol stone	11.83 ± 3.57	7.08 ± 2.31	31.20 ± 7.78[a]	22.15 ± 2.87[c]
Gastrin				
Control	17.83 ± 2.35	18.56 ± 5.77	17.42 ± 6.39	19.41 ± 4.58
Cholesterol stone	0.08 ± 0.03[c]	4.18 ± 2.87	2.16 ± 0.44[a]	18.92 ± 8.37
CCK-8				
Control	1970.33 ± 439.82	2353.32 ± 13.18	2100.27 ± 29.70	2107.77 ± 171.70
Cholesterol stone	2214.61 ± 174.96	2044.18 ± 147.03	2034.75 ± 138.39	2042.61 ± 265.01

[a]$P < 0.05$, [c]$P < 0.01$ *vs* the control group. VIP: Vasoactive intestinal peptide; CCK-8: Cholecystokinin octapeptide.

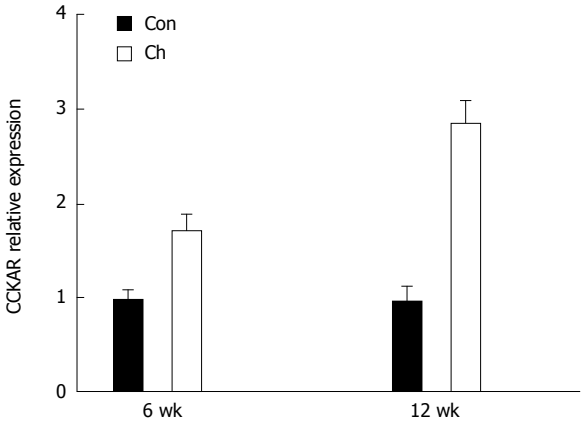

Figure 3 Cholecystokinin-A receptor mRNA expression levels in different groups. CCKAR: Cholecystokinin-A receptor.

The mechanism of cholesterol gallstone-causing diet-induced SOD has not been fully elucidated. Pang *et al*[19] considered that the potential mechanisms of hypercholesterolemia (HC)-induced SOD were the intracellular calcium overload and calcium oscillation abnormality. Szilvassy *et al*[21] suggested that the impairment of the SO relaxation function was related to the alteration of the nitric oxide signal caused by hypercholesterolemia and hypertriglyceridemia. SO motility was controlled by numerous neurotransmitters and gastrointestinal hormones[22]. The most important hormone in the regulation of SO function is CCK[23]. In normal conditions, CCK can contract the gallbladder smooth muscle and reduce tone in the SO to regulate bile flow from the liver through the bile duct into the duodenum. During fasting, hepatic bile enters the gallbladder for storage. Eating initiates gallbladder emptying by neural and hormonal (predominantly CCK) influences. We found serum CCK-8 level was not increased in the 9 and 12 wk groups, meaning that the gallbladder evacuation resistance increased during eating in these groups. Decreases in serum CCK-8 level can cause gallbladder bile stasis, increase the volume of the gallbladder, and induce SOD by increasing the tension of SO.

CCK modulates SO motility mainly *via* excitatory receptors on the smooth muscle and inhibitory receptors in the neural endings[24]. A study in cats[24] showed that the SO relaxation by CCK was abolished by complete denervation induced by tetrodotoxin. Beagle dogs after cholecystectomy[25] showed an increased CBD pressure, and SO relaxation response to CCK was weakened. These findings were thought to be due to destruction of neural pathways with the operation. In our study, we examined the expression of CCK-AR in the SO using quantitative real-time PCR for the first time. We found that the expression levels of *CCK-AR* mRNA increased in SO in the 6 and 12 wk groups. The increase in CCK-AR may result in changes in tension of SO, and, therefore, the excreting resistance of the bile may be enhanced significantly.

VIP, an alkaline intestinal peptide composed of 28 amino acids, can relax gallbladder smooth muscle and decrease gallbladder pressure. In the present study, serum VIP level in the 9 and 12 wk groups was higher than that of the control group. We restated the role of VIP in the formation of cholesterol gallstones that had been stressed in our previous study[12]. We found that the frequency of myoelectric activity decreased markedly in the 9 wk group, the amplitude of myoelectric activity decreased in the 9 and 12 wk groups, and the SO basal pressure and common bile duct pressure increased significantly in the 12 wk group. Elevation of VIP may play an important role in the mechanism of SOD.

Gastrin can increase the SO basal pressure amplitude[12]. Elevation of serum gastrin may be related to SOD with characteristics of high tension in patients with post-cholecystectomy pain[26]. However, we found that serum gastrin was decreased significantly in the 3 and 9 wk groups. This effect may have resulted from changes in diet and may not necessarily be related to the formation of gallstones.

In conclusion, we have indicated that a cholesterol gallstone-causing diet may induce SOD, the increasing tension of SO along with its decreasing activity may play an important role in cholesterol gallstone formation, and expression changes in CCK-AR in SO smooth muscle and serum CCK-8 and VIP may be important causes of SOD. The exact mechanism of cholesterol gallstone-causing diet-induced SOD needs further study. Control

of a cholesterol diet and regulation of SO motility are important in the prevention of cholesterol gallstone formation.

COMMENTS

Background

The sphincter of Oddi (SO) may play an important role in gallstone formation because it is the only way by which bile is discharged into the duodenum. However, there is limited research on how the motor function of the SO works and what mechanism by which stones are formed in the process of cholesterol gallstone formation.

Research frontiers

SO manometry is the only method to directly assess the motor function of the SO and is considered the gold standard for assessing SO dysfunction (SOD). However, manometry changes of SO in cholelithiasis are quite controversial. Another measurement reflecting SO function is recording the myoelectric activity of the SO.

Innovations and breakthroughs

This study showed that a cholesterol gallstone-causing diet can induce SOD. The increasing tension of the SO along with its decreasing activity may play an important role in cholesterol gallstone formation. Expression changes of cholecystokinin-A receptor in SO smooth muscle, serum vasoactive intestinal peptide, and cholecystokinin-octapeptide may be important causes of SOD.

Applications

The study results suggest that a cholesterol gallstone-causing diet can induce SOD, and disturbance of SO motility may play a role in gallstone formation. Control of a cholesterol diet and regulation of SO motility are important in the prevention of cholesterol gallstone formation.

Peer-review

This is an interesting paper. The authors describe the results of a basic study in a guinea pig model to investigate the role of SO motility in cholesterol gallbladder stone formation. They concluded that a cholesterol gallstone causing diet may induce increasing tension and decreasing activity of SO, and these effects could play an important role in cholesterol gallstone formation. This work represents a well-conducted basic study that contributes useful information about the role of SO motility in the process of cholesterol gallstone formation and confirms previous limited data that indicated a significant increase in the base pressure of the SO in rabbits with a cholesterol lithogenic diet.

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ORIGINAL ARTICLE

Case Control Study

Comprehensive risk assessment for early neurologic complications after liver transplantation

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Author contributions: Feng AC, Hsieh CB, Fan HL and Chen TW performed the research; Chung KP participated in the design of the study and performed the statistical analysis; Wu SY, Feng AC and Hsieh CB wrote the paper; all authors read and approved the final manuscript.

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Data sharing statement: Technical appendix, original data, and statistical code of manuscript are available from the corresponding author at albert0920@yahoo.com.tw.

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Abstract

AIM: To determine risk factors for early neurologic complications (NCs) after liver transplantation from perspective of recipient, donor, and surgeon.

METHODS: In all, 295 adult recipients were enrolled consecutively between August 2001 and February 2014 from a single medical center in Taiwan. Any NC in the first 30 d post-liver transplantation, and perioperative variables from multiple perspectives were collected and analyzed. The main outcome was a 30-d NC. Generalized additive models were used to detect the non-linear effect of continuous variables on outcome, and to determine cut-off values for categorizing risk. Risk factors were identified using multiple logistic regression analysis.

RESULTS: In all, 288 recipients were included, of whom 142 (49.3%) experienced at least one NC, with encephalopathy being the most common 106 (73%). NCs prolonged hospital stay (35.15 ± 43.80 d *vs* 20.88 ± 13.58 d, $P < 0.001$). Liver recipients' age < 29 or ⩾ 60 years, body mass index < 21.6 or >

27.6 kg/m^2, Child-Pugh class C, history of preoperative hepatoencephalopathy or mental disorders, day 7 tacrolimus level > 8.9 ng/mL, and postoperative intra-abdominal infection were more likely associated with NCs. Novel risk factors for NCs were donor age < 22 or ⩾ 40 years, male-to-male gender matching, graft-recipient weight ratio 0.9%-1.9%, and sequence of transplantation between 31 and 174.

CONCLUSION: NCs post- liver transplantation occurs because of factors related to recipient, donor, and surgeon. Our results provide a basis of risk stratification for surgeon to minimize neurotoxic factors during transplantation.

Key words: Risk; Liver transplantation; Neurotoxicity syndromes; Donor; Learning curve

Core tip: The study uses generalized additive models and logistic regression in statistics to control confounders in the case-control study. We identified 11 risk factors for early neurologic complication after transplantation. From liver recipients' perspective, age < 29 or ⩾ 60 years, body mass index < 21.6 or > 27.6 kg/m^2, Child-Pugh class C, history of preoperative hepatoencephalopathy or mental disorders, day 7 tacrolimus level > 8.9 ng/mL, and postoperative intra-abdominal infection were at risk. From donors' perspective, age < 22 or ⩾ 40 years, male-to-male gender matching, graft-recipient weight ratio 0.9%-1.9% was at risk. From surgeons' perspective, sequence of transplantation between 31 and 174 were at risk.

Wu SY, Chen TW, Feng AC, Fan HL, Hsieh CB, Chung KP. Comprehensive risk assessment for early neurologic complications after liver transplantation. *World J Gastroenterol* 2016; 22(24): 5548-5557 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i24/5548.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i24.5548

INTRODUCTION

Neurologic complications (NCs) are common after solid organ transplantation, especially liver transplantation[1]. While mortality rates decreased with advances in surgical technique and postoperative care in recent years, NCs continue to affect more than one-third of transplanted patients and cause significant morbidity, mortality, and prolonged hospital stay[2,3]. The liver recipient is vulnerable to NCs since many patients have preoperative hepatic encephalopathy, which is a well-known risk factor[3-6]. Moreover, the unfavorable condition of recipients including metabolic, nutritional, and electrolyte imbalances may predispose them to NCs[7,8]. Generally, NCs develop early, with more than 80% of patients developing NCs within 30 d after liver transplantation[6,9]. Encephalopathy is the most frequent etiology[3,5,9-12]. As it is multifactorial in nature, the transplant team should identify patients at risk and avoid predisposing variables during the perioperative period to minimize its incidence.

In the last decade, the risk factors and mechanism of NCs have been investigated in several studies, most of which were retrospective[3,6,9-12]. The majority of these focused on recipients' factors depending on timing of transplant, such as presence of hepatic encephalopathy, etiology of cirrhosis before transplant, hyponatremia, cerebrovascular insult during transplant, immunosuppressant toxicity and central nervous system infection after transplant. However, the nature of liver graft, donor-recipient matching, and experience of surgeon and transplant team also impact outcome, and few studies have investigated NCs after liver transplantation from these perspectives. No study showed how the accumulated experience of the transplant team influences NC development. Besides, the non-linear effect of certain continuous variables, such as age, body mass index (BMI) and graft-recipient weight ratio (GRWR), was not considered in the statistical analysis of prior studies. Our study aimed to identify new risk factors of 30-d NCs after liver transplantation from multiple perspectives. In particular, we performed statistical analyses to explore how the non-linear effect of certain continuous variables increase NC risk.

MATERIALS AND METHODS

Patients

From August 2001 to February 2014, 295 consecutive adult liver transplantation surgeries were performed in the Tri-Service General Hospital (Taipei, Taiwan). All causes of mortality were included in the study except for surgical mortality unrelated to NCs. All procedures were performed after approval of the Ethics Committee. The transplant team included 2 qualified transplant surgeons, 2 fellows and 2 senior residents. The immunosuppressant induction protocol consisted of intravenous methylprednisolone, starting with a large bolus dose during the anhepatic phase, followed by daily tapering off until postoperative day 5, and triple oral immunosuppressants (tacrolimus, mycophenolate mofetil, steroids). Serum trough levels of tacrolimus were checked daily in the first postoperative week to maintain a level of 6-10 ng/mL. If basiliximab was included, the first 20 mg were given during anhepatic phase and the second dose on postoperative day 4, along with halving the steroid dose. Diagnoses of NCs were primarily made by the transplant team based on clinical examination, and ancillary examinations such as computed tomography, magnetic resonance imaging, and electroencephalography. Neurologists were consulted for major NCs. Only new onset of

postoperative neurologic disorders was regarded as NCs. The clinical data and patients' outcomes were retrospectively reviewed from medical charts. The final follow-up was conducted until March 31, 2014.

Data collection

The medical records and transplant database were reviewed after approval of the Institutional Review Board I of Tri-Service General Hospital, National Defense Medical Center (TSGHIRB No.100-05-220). Data were collected from 4 major perspectives: recipient, donor, donor-recipient match, and surgeon. The recipient-related variables were demographic details, comorbidities other than liver diseases, primary liver disease requiring liver transplantation, preoperative Child-Pugh and Model for End-Stage Liver Disease (MELD) scores, any complications of end-stage liver disease, such as ascites, hepatic encephalopathy, variceal bleeding and hepatorenal syndrome. In addition, any mental disorders related to alcoholism or encephalopathy were recorded. The perioperative variables included intraoperative blood loss, length of the procedure, complex vascular anatomy, and splenectomy. The postoperative variables included dose and serum level of tacrolimus, inclusion of basiliximab, and blood chemistry parameters (ammonia, sodium, and magnesium) on postoperative day 7. In addition, any postoperative complications as acute rejection, intra-abdominal infection, or kidney injury requiring dialysis were recorded. The donor and donor-recipient matching-related variables were graft's type and weight, gender match, GRWR, and ABO compatibility. The surgeon variables were surgeon in charge, and the chronological sequence of transplantations.

Outcomes' evaluation

The primary outcome was occurrence of any NCs within 30 d after liver transplantation. We adapted the NC classification from Dhar *et al*[3] as follows: (1) encephalopathy: delirium, psychosis, or alteration of conscious level diagnosed after excluding specific lesions of the central nervous system; (2) seizures; (3) drug neurotoxicity: symptoms subsiding after reduction or discontinuation of immunosuppressants, with severity varying from tremors to imaging-confirmed posterior reversible encephalopathy syndrome; (4) cerebrovascular insults; (5) central nervous system infection; and (6) central pontine myelinolysis: acute para- or quadriparesis, dysphagia, dysarthria, diplopia, loss of consciousness with evident change in serum sodium levels. Patients experiencing "locked-in syndrome", that is, awake but unable to move or communicate, were categorized into "central pontine myelinolysis" if rapid changes in serum sodium level coexisted. If the definite etiology could not be determined, they were recorded separately.

Other outcome data were hospital days and complications other than NCs during the first month. In addition, the complications were classified into minor and major according to the clinical finding and its severity. Minor complications included those that improved spontaneously without sequela within 1 mo, while the others causing functional deficit, brain damage or death were considered as major complications.

Statistical analysis

Statistical analysis was performed using the R 3.1.0 software (R Foundation for Statistical Computing, Vienna, Austria). A P-value of < 0.05 was considered statistically significant.

Descriptive statistics were used to express data as mean ± SD for continuous variables, and frequency and percentage for categorical variables. Differences between the two groups, patients developing NCs and those who did not, were analyzed using Wilcoxon's rank-sum test for continuous variables, and Fisher's exact test for categorical variables.

Some continuous variables had non-linear effect, such as age and BMI. The regression model may have described poor correlation between these variables and the outcome since linearity was usually assumed during the analysis. Generalized additive models (GAMs)[13] for binary response (patients developing NCs and those who did not) were fitted to detect the potential nonlinear effects of continuous covariates; if nonlinearity existed, appropriate cut-off point(s) for discretizing the continuous covariate were selected in the GAM plots. This procedure was carried out using the vgam function (with the default values of smoothing parameters) of the VGAM package in R.

Multiple logistic regression analysis was conducted by fitting a generalized linear model to estimate the effects of predictors on the occurrence of NCs. First, all variables, donor-recipient gender match combinations, and new categorical variables obtained from previous cut-off points for discretizing continuous variables by GAM were selected. Next, a step-wise variable selection procedure went through iteration between the forward and backward steps with both the significant levels for entry and for stay set to 0.15 for being conservative. Then, the best candidate final logistic regression model was identified manually by dropping the covariates with P value > 0.05 one at a time until all regression coefficients were significantly different from 0. The final fitted logistic model was assessed with 3 goodness of fit measures: the estimated area under the receiver operating characteristic curve ($\geqslant$ 0.7 acceptable), adjusted generalized R^2 ($\geqslant$ 0.30 acceptable), and the Hosmer-Lemeshow test ($P \geqslant$ 0.05, larger means better fit). In addition, the variance inflation factor was checked for multicollinearity (no more than 10 for continuous covariates and 2.5 for categorical covariates).

RESULTS

Among the 295 patients, 7 with surgical mortality

Table 1 Baseline characteristics of the 288 consecutive liver transplantation patients

Recipient variables	
Demographics	
Age (yr)	52.3 ± 9.81
Gender (M/F)	213/75
BMI (kg/m^2)	24.4 ± 3.72
Primary diagnosis, *n* (%)	
Hepatitis B	175 (60.8)
Hepatitis C	73 (25.3)
Alcoholic liver disease	77 (26.7)
HCC	138 (47.9)
Severity of liver disease	
Child-Pugh score	10.0 ± 2.49
MELD score	15.0 ± 9.01
Complication, *n* (%)	
Hepatic encephalopathy	132 (45.8)
Ascites	189 (65.6)
Variceal bleeding	114 (39.6)
Comorbidities, *n* (%)	
Diabetes mellitus	103 (35.7)
Hypertension	52 (18.1)
Uremia	14 (4.9)
Mental disorders	99 (34.4)
Blood test before transplant	
Glucose (mg/dL)	129 ± 76.07
Albumin (g/dL)	3.08 ± 0.66
Creatinine (mg/dL)	1.22 ± 1.53
INR	1.62 ± 0.99
T. bilirubin (mg/dL)	7.78 ± 11.19
Platelet (× 10^3/μL)	85.4 ± 53.85
Ammonia (μg/dL)	138.0 ± 97.05
Perioperative variables	
Blood loss (mL)	2896 ± 3409
Operation time (min)	575 ± 122.7
Splenectomy, *n* (%)	87 (30.2)
Complex vascularity, *n* (%)	75 (26.0)
Postoperative variables	
Tacrolimus dose (mg/d)	3.4 ± 1.67
Basiliximab, *n* (%)	51 (18)
Serum level - Day 7	
Tacrolimus level (ng/mL)	7.4 ± 6.24
Ammonia (μg/dL)	69.6 ± 130.8
Sodium (mmol/L)	136 ± 4.92
Magnesium (mEq/L)	1.73 ± 0.34
Donor variables	
Age (yr)	33 ± 11.21
Male gender, *n* (%)	171 (59.4)
Graft weight (g)	872 ± 357.88
Graft type	95/47/142/4
(whole/left/right/S67), *n* (%)	(33.0/16.3/49.3/1.4)
Donor-recipient matching	
GRWR (%)	1.34 ± 0.57
ABO incompatible, *n* (%)	9 (3.1)
Gender match	128/44/86/30
(MM/MF/FM/FF), *n* (%)	(44.4/15.3/29.9/10.4)
Surgeon variables	
Surgeon A/B/C, *n* (%)	234/51/3 (81/18/1)
Sequence of transplantation	1-288

BMI: Body mass index; HCC: Hepatocellular carcinoma; MELD: Model for end-stage liver disease; GRWR: Graft-recipient weight ratio.

unrelated to NCs were excluded: 2 failed to awake due to irreversible brain damage secondary to fulminant hepatitis, and 5 experienced primary graft failure. Finally, 288 patients were enrolled in this study, with 95 receiving deceased donor liver transplantation (DDLT) and 193 receiving living donor liver transplantation (LDLT). Baseline characteristics of the study population are shown in Table 1.

Table 2 Type of complication after liver transplantation *n* (%)

	Event	Minor/major
Neurologic complications	145 (100)	97 (67)/48 (33)
Encephalopathy	106 (73)	88 (83)/18 (17)
Delirium	75 (52)	64 (85)/11 (15)
Psychosis	5 (4)	4 (80)/1 (20)
Change in consciousness	26 (18)	20 (77)/6 (23)
Seizures	10 (7)	0 (0)/10 (100)
Cerebrovascular events	5 (4)	0 (0)/5 (100)
Drug neurotoxicity	10 (7)	5 (50)/5 (50)
Locked-in syndrome	12 (8)	4 (33)/8 (67)
Central pontine myelinolysis	2 (1)	0 (0)/2 (100)
Other complications		
Acute kidney injury	1	
Intra-abdominal infection	9	
Graft failure	2	
Reoperation	31	
Acute rejection	61	
Hepatitis B recurrence	10	
Tuberculosis infection	8	
Cytomegalovirus infection	7	
Total complications	85	

The outcome data are shown in Table 2. One hundred and forty two patients experienced 145 events of NCs, with an overall incidence of 49.3%. The most frequent events were encephalopathy (n = 106, 73%), including delirium (n = 75, 52%), conscious change (n = 26, 18%) and psychosis (n = 5, 4%). The average hospital stay was significantly longer in those with postoperative NCs (35.15 ± 43.80 d *vs* 20.88 ± 13.58 d, P < 0.001).

Variables associated with NCs

The differences between the patients with NC or not are shown in Table 3. Both groups were similar in demographics of recipient and donor, liver graft type, surgeon and sequence of transplantation. For primary diagnosis, alcoholic liver cirrhosis was more common in the NC group (P = 0.001), while hepatocellular carcinoma was prevalent in the control group (P < 0.001). The NC group included patients with more severe liver disease before transplant, more preoperative mental disorders and hepatic encephalopathy, more intraoperative complex vascular anatomy, higher day 7 tacrolimus level, more postoperative complications of acute rejection, intra-abdominal infection, and kidney injury requiring dialysis.

GAMs were fitted to continuous variables with potential non-linear effect on outcome. The selected GAM plots for continuous variables on the NC group are shown in Figure 1. According to the GAM plots, the following scales of variables were associated with higher probability of NCs: age < 29 or ⩾ 60, recipient BMI < 21.6 or > 27.6 kg/m^2, Child-Pugh score >

Table 3 Comparison of variables from multiple perspectives between the neurologic complications and control groups

	NC (n = 142)	No NC (n = 146)	P value
Recipient variables			
Preoperative			
Age (yr)	51.75 ± 10.51	52.77 ± 9.09	0.567
Gender (M/F)	109/33	104/42	0.347
BMI (kg/m^2)	24.65 ± 4.25	24.26 ± 3.11	0.617
Hepatitis B	86	89	1.000
Hepatitis C	30	43	0.136
Alcoholic liver disease	50	27	0.001
HCC	51	87	< 0.0001
Child-Pugh score	10.26 ± 2.26	8.27 ± 2.32	< 0.0001
MELD score	20.3 ± 9.4	14.0 ± 7.4	< 0.0001
Hepatic encephalopathy	91	41	< 0.0001
Variceal bleeding	51	63	0.229
Ascites	106	83	0.002
Mental disorder	66	33	< 0.0001
Serum Albumin (g/dL)	2.95 ± 0.57	3.21 ± 0.72	0.01
Serum T. bilirubin (mg/dL)	10.91 ± 13.16	4.74 ± 7.79	< 0.0001
Perioperative			
Blood loss (mL)	3329 ± 3953	2474 ± 2728	0.081
Operation time (min)	558.98 ± 99.63	579.9 ± 127.0	0.160
Complex vascularity	45	30	0.033
Postoperative			
Day 7 tacrolimus level (ng/mL)	8.23 ± 7.42	6.54 ± 4.69	0.023
Acute rejection	38	23	0.030
Intra-abdominal infection	32	12	< 0.0001
Kidney injury requiring dialysis	18	7	0.021
Donor variables			
Donor age (yr)	32.5 ± 11.4	33.6 ± 11.0	0.234
Donor gender (M/F)	89/53	82/64	0.282
Graft type (whole/left/right/S67)	45/21/74/2	50/26/68/2	0.827
Donor-recipient matching			
GRWR	1.33 ± 0.56	1.35 ± 0.57	0.726
Surgeon variables			
Surgeon A/B/C	116/25/1	118/26/2	0.855
Sequence of transplantation	95.35 ± 69.66	108.12 ± 74.41	0.186

NCs: Neurologic complications; HCC: Hepatocellular carcinoma; MELD: Model for end-stage liver disease; GRWR: Graft-recipient weight ratio; BMI: Body mass index.

9, MELD score between 11 and 20, serum glucose > 8 mmol/L, serum albumin between 2.9 and 4.2 g/dL, serum creatinine between 0.6-1.6 mg/dL, the logarithm of INR > 0.3, platelet count < 70 × 10^3/μL, serum ammonia > 268 μg/dL, day 7 tacrolimus level > 9 ng/mL, day 7 serum ammonia level > 98 μg/dL, day 7 serum sodium level > 143 mmol/L, day 7 serum magnesium level > 1.8 mEq/L, donor age < 22 or ⩾ 40 years, GRWR between 0.9% and 1.9%, and sequence of transplantation between 31 and 174. New categorical variables for regression analysis were obtained after grouping these continuous variables.

After stepwise multiple logistic regression analysis, 12 independent variables were identified as significant in the final logistic regression model (Table 4). Eleven positive predictors of NCs were: recipient age < 29 or ⩾ 60 years, BMI < 21.6 or > 27.6 kg/m^2, Child-Pugh score > 9, preoperative hepatic encephalopathy, history of mental disorder, day 7 tacrolimus level > 8.9, postoperative intra-abdominal infection, donor age < 22 or ⩾ 40 years, male-to-male gender match, GRWR between 0.9% and 1.9%, and sequence of transplantation between 31 and 174. On the other hand, recipients with history of variceal bleeding were less likely to develop NCs (OR = 0.431; 95%CI: 0.221-0.821). The assessment of final logistic regression model showed fair goodness-of-fit (area under the receiver operating characteristic curve = 0.8553 > 0.7 with 95%CI: 0.8119-0.8988; adjusted R^2 = 0.471 > 0.3; Hosmer-Lemeshow goodness-of-fit test P = 0.1446 > 0.05). Furthermore, variance inflation factors for each covariate in the selected final logistic regression model were between 1.075 and 1.582, indicating no multicollinearity.

DISCUSSION

Our results confirmed the prevalence of NCs following liver transplantation, with three-quarters of patients developing encephalopathy. The risk factors were not only from recipient's perspective, but also from perspectives of donor, donor-recipient match and surgeon. Since randomization of subjects is hardly possible in critical conditions as those requiring liver transplantation, studies in this area almost have case-control design. The novelty we have in this study came from better control of confounding effects by flexible statistical tools. The GAM plots enabled more proper stratification of continuous variables on outcome, and the regression analysis controlled the confounding bias by considering multiple covariates all at a time.

The incidence of NCs in the study was relatively higher than in other reports (49.3% *vs* 20%-30%) because of more diagnoses of minor encephalopathy (88/145, 60.7% of overall NC events). In fact, the diagnostic criteria for encephalopathy are not universal among physicians, especially for minor degrees. In our institution we tend to broaden the diagnostic criteria to include any transient delirium, psychosis or consciousness' level change. This enables us to correct metabolic disorders and use immunosuppressants more properly. Our categorical system here is limited by not differentiating anoxic, septic or metabolic etiologies[14] accounting for intra-abdominal infection as an independent risk factor (OR = 5.193, 95%CI: 2.114-13.67).

Preoperative hepatic encephalopathy and mental disorders significantly increased the risk of NCs, with an OR 2.432 and 2.517, respectively. Hepatic encephalopathy, both episodic and active before the operation, is a well-known risk factor[3,4,6,12,14]. It is hypothesized that excess serum ammonia in end stage liver disease interferes with cerebral metabolism, and the condition is not immediately reversed after

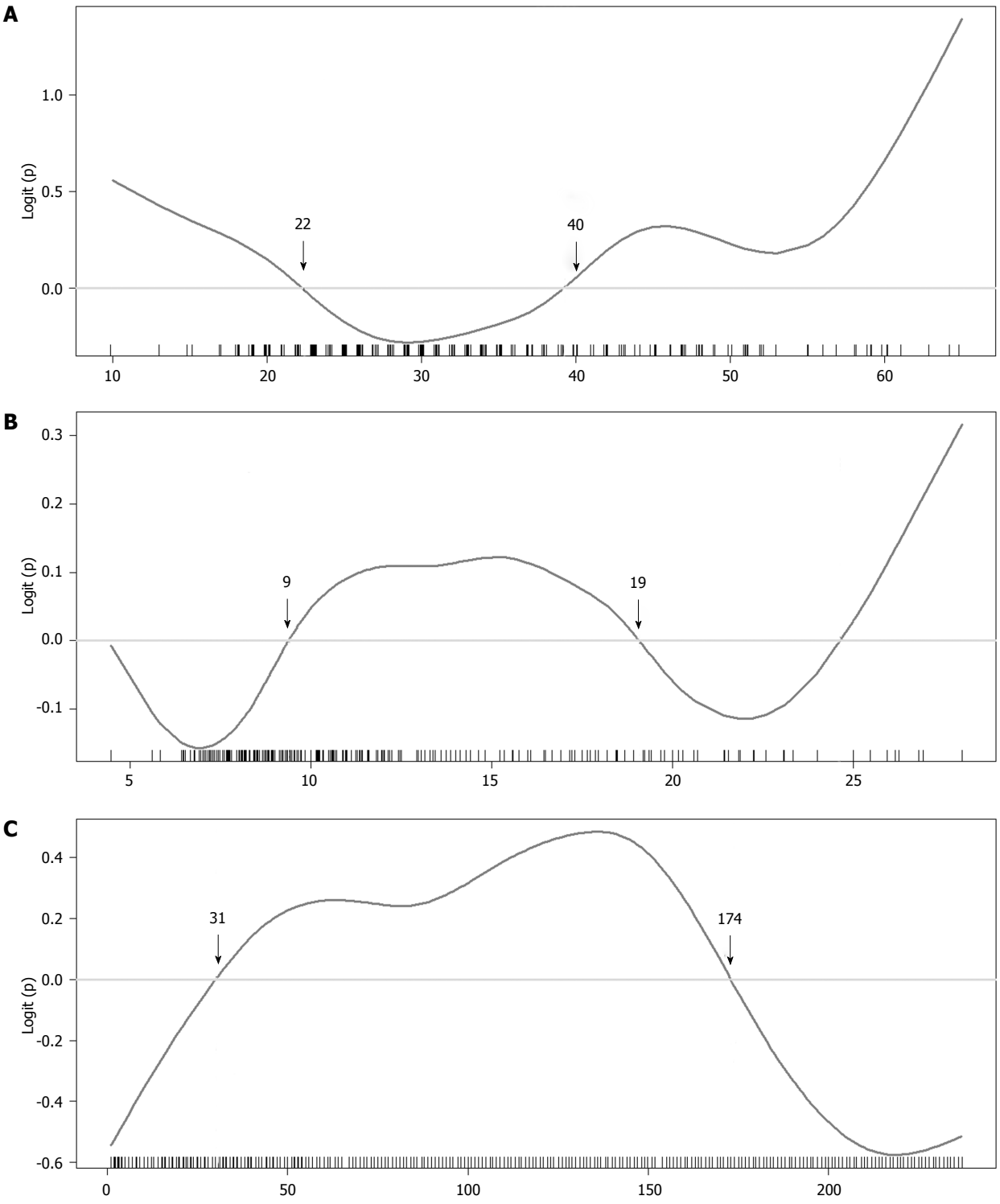

Figure 1 Influence of continuous covariates on neurologic complications resulting from fitting a generalized additive model to the data. A: Donor age; B: Graft-recipient weight ratio; C: Sequence of transplantation.

transplantation. Several investigators have emphasized the importance of comprehensive preoperative neurologic examinations including neuropsychiatric and neuromuscular assessments.

In addition to hepatic encephalopathy, chronic alcohol abuses also contribute to neurotoxicity, which impairs cognitive function especially memory. Alcoholism also renders patients at risk for thiamine deficiency. A prospective study that performed neuropsychological assessments before and after liver transplantation found that recipients with alcoholic etiology had poorer cognitive indexes in memory; in addition, a multivariate analysis determined that alcohol etiology, diabetes mellitus, and hepatic encephalopathy were predictors of poor global cognitive function after transplantation[15]. However,

Table 4 Multiple logistic regression model for neurologic complications

	OR	95%CI	*P* value
Recipient variables			
Preoperative			
Age < 29 or ⩾ 60 yr	2.071	1.024-4.272	0.045
BMI < 21.6 or > 27.6 kg/m^2	1.877	1.007-3.552	0.049
Child-Pugh score > 9 (Class C)	1.509	1.288-1.790	< 0.001
Hepatic encephalopathy	2.432	1.232-4.860	0.011
Variceal bleeding	0.431	0.221-0.821	0.012
Mental disorder	2.517	1.279-5.064	0.008
Postoperative			
Day 7 tacrolimus level > 8.9 ng/mL	1.131	1.068-1.205	< 0.001
Intra-abdominal infection	5.193	2.114-13.67	< 0.001
Donor variable			
Donor age < 22 or ⩾ 40 yr	2.245	1.207-4.271	0.012
Donor-recipient matching			
Male to male gender match	2.36	1.266-4.506	0.008
0.9% < GRWR < 1.9%	1.95	1.069-3.624	0.032
Surgeon variable			
31 ⩽ Sequence of transplantation < 174	2.773	1.479-5.363	0.002

NCs: Neurologic complications; BMI: Body mass index; MELD: Model for end-stage liver disease; GRWR: Graft-recipient weight ratio.

in line with the result of recent studies on LDLT[10,14,16], the current study, using regression analysis adjusted by other covariates, did not find alcoholic liver disease to be a predictor of NCs. The discrepancy may be related to the multifactorial nature of neurologic complications, and it is difficult to attribute to a specific cause of NC in patients with alcoholic liver disease. As a result, clinicians believe that hepatic encephalopathy, which is prevalent in cirrhotic patients, could be the most common cause of NC. A controlled study with the exact definition of alcohol abuse, complete neurologic examination before and after transplantation, standard postoperative care, and exclusion of postoperative complications confounding the result is needed to answer the question.

On the other hand, recipients with history of variceal bleeding were not likely to have NCs after controlling of potential confounders in the regression model. It was somewhat difficult to explain. One possible reason was that our recipients with variceal bleeding tended to receive living donor liver transplantation earlier than those without did, which enabled transplant proceeding under less severe liver disease. Another reason might be related to the shunt ligation procedure which we often done during transplantation blocking portosystemic encephalopathy.

Moreover, patients with NCs had more severe liver disease before transplant. Child-Pugh class C was an independent risk factor, with an odds ratio of 1.509 (95%CI: 1.288-1.790), while MELD score was not when adjusted by serum creatinine level and other factors in regression analysis. These results were consistent with data obtained in previous studies. In a retrospective study by Dhar *et al*[3] investigating factors associated with NCs, preoperative Child-Pugh Class C was a significant variable in the univariate analysis, while only active preoperative hepatic encephalopathy was significant in the multivariate analysis. Another retrospective study by Kanwal *et al*[17] used age, sex and era-matched control group to identify risk factors of post-transplant mental status change. MELD score > 15 was an independent risk factor in both univariate and multivariate analyses and was one of the four factors included in the prediction model. These results are likely to be related to encephalopathy precipitated by higher serum level of endogenous neurotoxic substances (including ammonia) in patients with severe liver disease. Besides, the frequent changes in electrolyte levels, malnutrition and metabolic disorders in decompensated cirrhosis may result in an unfavorable postoperative environment, rendering liver recipients vulnerable to postoperative neurologic disorders.

Age < 29 or ⩾ 60 years and BMI < 21.6 or ⩾ 27.6 kg/m^2 showed increased probability of NCs in the GAM plot, and both were independent predictors in multiple logistic regression model, with odds ratios of 2.071 and 1.877, respectively (Table 4). Advanced age, preoperative cognitive impairment and multiple medical comorbidities were known risk factors for postoperative delirium after various procedures in several studies[18-20]. We believe that age ⩾ 60 years had greater impact on NCs since the mean recipients' age in our study was 52.3 ± 9.81 years, without pediatric recipients.

Underweight and overweight liver recipients had a significant risk for NCs. Extreme BMI values (< 18.5 and ⩾ 40 kg/m^2) are known risk factors for mortality after liver transplantation[21]. Patients are also more likely to develop infectious complications owing to malnutrition, as well as prolonged treatment time, and NCs secondary to of vitamins' and trace elements' deficiencies[22]. Early diagnosis and prompt treatment of nutritional deficits before transplantation may ameliorate encephalopathy and optimize transplant outcome[22,23].

In the GAM plot for postoperative variables, we found that day 7 tacrolimus level > 8.9 ng/mL, serum ammonia level > 98 μg/dL, serum sodium level > 143 mmol/L, serum magnesium level > 1.8 mEq/L were associated with higher probabilities for NCs. In the final logistic regression model, only day 7 tacrolimus level was an independent variable. This is in line with prior study[3].

Neurotoxicity during induction of immunosuppressants, mostly induced by calcineurin inhibitors (CNIs), is common in the early postoperative period. Clinical manifestations may vary from tremors, headache, and visual disturbances to altered mental status. In addition, seizures after transplantation are most often caused by drug neurotoxicity. The incidence is associated with high serum levels of CNIs while the occurrence is not excluded by normal serum CNI level. Coexisting hypomagnesemia and hypocholesterolemia

are risk factors. Rarely, patients using CNIs have posterior reversible encephalopathy syndrome, a radiographic diagnosis characterized by reversible vasogenic edema of the white matter involving the posterior circulation territory on serial magnetic resonance imaging. It is likely to occur in patients with concomitant alcoholic liver disease and infection/sepsis. Management is mainly dose reduction and shift to an immunosuppressant with another mechanism of action; however, these patients may be at risk of acute rejection from the lower maintenance dose[24]. The process of optimizing immunosuppression, aiming to minimize graft rejection and avoid neurotoxicity, may need multidisciplinary teamwork and accumulated experience.

From the donor perspective, variables of graft type, donor age, donor gender, and GRWR were not significantly different between the NC and control groups. While age and GRWR had a biphasic effect on the GAM plots (Figure 1), we adjusted the cut-off values (donor age < 22 or > 40 years, and 0.9% < GRWR < 1.9%) and found that both variables were predictors in the regression analysis. In the literature, few studies investigated the influence of donor and donor-recipient match factors on NCs. Study from the SRTR database have shown an increased risk of graft failure with donor age > 40 years[25]. Prior studies on NC risk factors including donor age and GRWR showed no significant difference of these variables[5,11]. In contrast, our study indicated that risk of NC depends on either extreme donor age, or improper GRWR. In the GAM plots, we could see the probability of NCs decreasing when GRWR was above 1.9, which could suggest better detoxification capabilities of larger grafts. Besides, small size grafts are not likely to increase NC risk, suggesting non-inferiority of small graft when graft inflow is properly controlled[26].

Male-to-male gender match was another independent risk factor for NCs in this study. The association between donor-gender match and neurologic complications has not been reported previously. The impact of gender mismatch on the outcome of liver transplantation is still controversial. Several studies demonstrated that female-to-male gender match is associated with negative outcomes in kidney, heart, lung, and liver transplantations, possibly due to the effect of estrogen and the relative small-for-size of female grafts[27-30]. It is unclear whether other confounding variables specific to male gender play a role, such as prevalent alcoholic liver diseases. Further prospective, controlled studies are needed to confirm the gender match effect on NCs.

The sequence of transplantation was a notable finding. The risk of NCs decreased after 174 transplantations by the team, and the order between 31 and 174 was an independent risk factor for NC development. The lower risk in the first 30 cases could be related to strict patient selection and relatively conservative strategy of immunosuppression. Learning curve effect in liver transplantation has been described, which is affected by the volume of the center or the year of transplant[31-34]. For successful liver transplantation, multidisciplinary team work is needed. The improvement is important, not only in the surgical technique but also in donor selection, timely decision for surgery, standardized postoperative care and optimal immunosuppression. Compared to graft survival rate commonly used in studies with a learning curve, NCs are more likely to be related to non-operative factors[35,36]. It is the environment influenced by perioperative risk factors from all perspectives that results in complications. To avoid NCs, the transplant team may need risk stratification during patient selection and donor matching, correcting risk factors before the operation as much as possible, and should remain watchful during the perioperative period. Ideal immunosuppression aims at minimizing acute rejection and avoids neurotoxicity. It is crucial for the transplant surgeon to recognize this process of development and to conduct interdisciplinary learning as well as continuously improving the patient care quality of the team.

This study has limitations owing to its retrospective design. First, the complications included were those that were identified and reported by the clinician. While serious complications are rarely excluded, clinicians may have missed minor complications that resolved spontaneously without treatment. Second, NCs often manifests as one or more signs, but it is possible that only the most serious ones were documented, leading to misclassification. Third, patients in the control group might have shared features and exposures with those in the NC group. Even if we have considered as many potential important variables as we could, residual confounders are still possible. Despite these limitations, the risk factors identified in the study could be subjective for validation in further prospective, randomized studies.

In conclusion, NCs after liver transplantation occur frequently and affect almost half of liver recipients, with encephalopathy being the most common (73%). In this study, we identified 11 risk factors for neurologic complications. From the recipient's perspective, age < 29 or ⩾ 60 years along with BMI < 21.6 or > 27.6 kg/m^2 significantly increased NC risk, and Child-Pugh score > 9, hepatic encephalopathy, mental disorder, 7-d tacrolimus level > 8.9 ng/mL, intraabdominal infection were complementary to previous studies. Patients with history of variceal bleeding were less likely to develop NCs. Novel risk factors from donor's and surgeon's perspective were donor age < 22 or ⩾ 40 years, male-to-male gender match, GRWR between 0.9 and 1.9 and sequence of transplantation between 31 and 174. Our results provide a basis for risk stratification, which would enable transplant surgeons to weigh the risk during patient selection, control unfavorable factors before operation, avoid neurotoxicity during perioperative period, and perform active surveillance

after operation. The manner in which the experience of transplant team influences NCs should also be taken into account by the team leader or health care manager who would allow the conduction of interdisciplinary education as well as strategies for continuous quality improvement.

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COMMENTS

Background

Neurologic complications (NCs) are common after liver transplantation and result in significant morbidity and mortality. Encephalopathy, the most frequent etiology, is multifactorial in nature. Surgeons should identify patients at risk and avoid predisposing variables during the perioperative period to minimize their incidence. However, known risk factors have been mainly investigated from the recipient's perspective.

Research frontiers

The authors have considered various factors from recipient, donor, donor-recipient matching, and surgeon perspectives that had potential impact on early NC after liver transplantation. They had better control of confounders from the case-control study design using more flexible statistical tools, including generalized additive models for variables, which had a nonlinear effect on outcome.

Innovations and breakthroughs

In addition to risk factors from the recipient perspective that were complementary to those in the literature, novel risk factors discovered in this study were donor age (< 22 or ⩾ 40 years), male-to-male gender matching, graft-recipient weight ratio 0.9%-1.9%, and sequence of transplantation between 31 and 174.

Applications

The results provide a basis for risk stratification for surgeons to minimize neurotoxic factors during transplantation. In addition, the transplant team leader should be aware of the higher risk of neurologic complication during the team's earlier experiences to conduct interdisciplinary education and quality improvement program.

Peer-review

The manuscript is clearly written to describe the results with pretty good discussion. The method section is really well written as a clinical report and can be one of the greatest examples for physician-scientists.

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ORIGINAL ARTICLE

Case Control Study

Relationships between cell cycle pathway gene polymorphisms and risk of hepatocellular carcinoma

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Abstract

AIM: To investigate the associations between the polymorphisms of cell cycle pathway genes and the risk of hepatocellular carcinoma (HCC).

METHODS: We enrolled 1127 cases newly diagnosed with HCC from the Tumor Hospital of Guangxi Medical University and 1200 non-tumor patients from the First Affiliated Hospital of Guangxi Medical University. General demographic characteristics, behavioral information, and hematological indices were collected by unified questionnaires. Genomic DNA was isolated

from peripheral venous blood using Phenol-Chloroform. The genotyping was performed using the Sequenom MassARRAY iPLEX genotyping method. The association between genetic polymorphisms and risk of HCC was shown by *P*-value and the odd ratio (OR) with 95% confidence interval (CI) using the unconditional logistic regression after adjusting for age, sex, nationality, smoking, drinking, family history of HCC, and hepatitis B virus (HBV) infection. Moreover, stratified analysis was conducted on the basis of the status of HBV infection, smoking, and alcohol drinking.

RESULTS: The HCC risk was lower in patients with the *MCM4* rs2305952 CC (OR = 0.22, 95%CI: 0.08-0.63, *P* = 0.01) and with the *CHEK1* rs515255 TC, TT, TC/TT (OR = 0.73, 95%CI: 0.56-0.96, *P* = 0.02; OR = 0.67, 95%CI: 0.46-0.97, *P* = 0.04; OR = 0.72, 95%CI: 0.56-0.92, *P* = 0.01, respectively). Conversely, the HCC risk was higher in patients with the *KAT2B* rs17006625 GG (OR = 1.64, 95%CI: 1.01-2.64, *P* = 0.04). In addition, the risk was markedly lower for those who were carriers of *MCM4* rs2305952 CC and were also HBsAg-positive and non-drinking and non-smoking (*P* < 0.05, respectively) and for those who were carriers of *CHEK1* rs515255 TC, TT, TC/TT and were also HBsAg-negative and non-drinking (*P* < 0.05, respectively). Moreover, the risk was higher for those who were carriers of *KAT2B* rs17006625 GG and were also HBsAg-negative (*P* < 0.05).

CONCLUSION: Of 12 cell cycle pathway genes, *MCM4*, *CHEK1* and *KAT2B* polymorphisms may be associated with the risk of HCC.

Key words: Cell cycle pathway genes; Hepatocellular carcinoma; Single nucleotide polymorphism; Case-control study; Genetic susceptibility

Core tip: We analyzed the effects of polymorphisms of 12 cell cycle pathway genes on the risk of hepatocellular carcinoma (HCC) in a large population of 1019 HCC cases and 1138 controls. The results suggest that *MCM4* rs2305952 CC and *CHEK1* rs515255 TC, TT, TC/TT may be significantly associated with a decreased risk of HCC. *KAT2B* rs17006625 GG may increase the risk of HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a serious threat to human health worldwide. It is the fourth most common cancer and the second leading cause of cancer death, with nearly 746000 deaths per year[1]. The incidence of this fatal disease continues to increase. HCC occurrence and development are related to environmental factors, such as infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), cigarette smoking, and alcohol consumption, as well as genetic susceptibility[2-4]. Many studies strongly support that single nucleotide polymorphisms (SNPs) of a variety of genes are associated with HCC[5-7]. However, the genetic mechanism underlying the inherited component of HCC is still not fully understood.

The cell cycle comprises the events that result in the formation of two daughter cells through division of the parent cell. Cell cycle progression, including cell division, is influenced by three different types of molecules: cyclin, cyclin-dependent kinases, and cyclin kinase inhibitors[8]. The associations between the genetic susceptibility of genes which regulate the cell cycle and the risk of cancer are well known. For instance, a polymorphism of the *p27* generates an increased risk of squamous cell carcinoma of the head and neck[9], while polymorphisms of *p27* and *p21* are associated with a significantly increased risk of HCC[10]. Other cell cycle pathway genes implicated in cancer include *cyclinD1*[11], *p53*[12], *CHEK2*[13] and *P21*[14].

During the last several decades, an increasing number of studies have shown an association between genetic variants, mainly in the form of SNPs, and the risk of cancer, including breast[15], colorectal[16], cervical, and vulvar cancers[17], and HCC[18]. Despite investigations into the association of polymorphisms in cell cycle pathway genes with cancer susceptibility[19,20], in the case of HCC this association remains unclear. Therefore, in this hospital-based study we investigated the associations between the polymorphisms of SNPs in cell cycle pathway genes and the risk of HCC.

MATERIALS AND METHODS

Study population

For this case-control study, 2327 subjects were consecutively recruited from June 2007 to December 2013. The 1127 HCC patients were from the Tumor Hospital of Guangxi Medical University and were newly diagnosed with HCC based on biochemical (α-fetoprotein > 20 μg/L) and histopathological examinations. None had undergone radiotherapy or chemotherapy before blood sampling. The 1200 controls from the First Affiliated Hospital of Guangxi Medical University consisted of non-tumor patients admitted within the same period of time. Informed

Table 1 Summarized information of selected single nucleotide polymorphisms in cell cycle pathway genes

Genes	SNPs	Chromosome (position)	Allele	MAF (hapmap-HCB)
MCM4	rs2305952	8 (47962049)	C/T	C = 0.18
YWHAB	rs2425675	20 (44906293)	A/G	A = 0.20
CDKN2A	rs3088440	9 (21968160)	A/G	A = 0.08
TGFB3	rs3917148	14 (75980178)	A/C	C = 0.10
RBL2	rs3929	16 (53490396)	C/G	C = 0.20
RAD21	rs6987652	8 (116870042)	A/G	A = 0.12
SMAD3	rs11556090	15 (67194045)	A/G	G = 0.09
	rs8025774	15 (67190938)	C/T	C = 0.45
KAT2B	rs17006625	3 (20119604)	A/G	G = 0.14
	rs4858770	3 (20152931)	C/T	T = 0.47
MCM7	rs2070215	7 (100099174)	A/G	G = 0.29
	rs2261360	7 (100095370)	A/C	A = 0.37
CDKN1A	rs3176320	6 (36679011)	A/G	G = 0.17
CDC25C	rs3734166	5 (138329634)	A/G	G = 0.38
CHEK1	rs515255	11 (125627250)	C/T	T = 0.44

MAF (minor allele frequency) was derived from HCB population in HapMap website (http://hapmap.ncbi.nlm.nih.gov/). SNPs: Single nucleotide polymorphisms.

consent was obtained from all participants, who also agreed to truthfully complete the questionnaires.

Information and sample collection

General demographic and behavioral information, hematological indices, and data on the patients' age, sex, nationality, drinking habit, smoking habit, HBV infection, and family history of HCC were obtained in face-to-face interviews by trained investigators. Peripheral venous blood was collected in a vacuum EDTA anticoagulant tube from each participant. Genomic DNA was extracted using a standard phenol-chloroform extraction method and stored at -80 ℃.

SNP selection

From the GEO database (https://www.ncbi.nlm.nih.gov/geo/), we found three sets of whole genome expression microarray data which were related to HCC (GSE14520, GSE25097, and GSE12941). A total of 3826 different genes were selected using SPSS 16.0 software (SPSS Inc., Chicago, IL, United States) ($P < 0.05$). Gene ontology classification and pathway enrichment analysis were performed by blast2GO and DAVID (https://david.ncifcrf.gov/) and 40 cell cycle pathway genes involved in the cellular process were chose. The genotype information was downloaded from Hapmap website (http://hapmap.ncbi.nlm.nih.gov/), and functional SNPs were selected using Haploview 4.2 software (Cambridge, MA o2141, United States) based on a function prediction website (http://snpinfo.niehs.nih.gov/snpfunc.htm). Referring to the existing literature on these SNPs with HCC, 15 SNPs in 12 genes (*MCM4* rs2305952, *YWHAB* rs2425675, *CDKN2A* rs3088440, *TGFB3* rs3917148, *RBL2* rs3929, *RAD21* rs6987652, *SMAD3* rs11556090, rs8025774, *KAT2B* rs17006625, rs4858770, *MCM7* rs2070215, rs2261360, *CDKN1A* rs3176320, *CDC25C* rs3734166, and *CHEK1* rs515255) were selected in this study. Information of selected SNPs is shown in Table 1.

SNP genotyping

Before genotyping, each DNA sample was quantified using a UV-Vis spectrophotometer Q5000 (Quawell Technology, Inc., United States) and diluted to a final concentration of 50 ng/μL. SNP genotyping was performed using a MassARRAY system (Sequenom, San Diego, CA, United States) and a matrix-assisted laser desorption ionization-time of flight mass spectrometry method according to the manufacturer's instructions. Primers for PCR and extension were designed using the Assay Designer software package (Sequenom). For quality control, 5% of the samples were randomly chosen and genotyped twice for each locus. Among the 1127 patient samples and 1200 control samples, genotyping was successful for all 15 SNPs in both groups, with a success rate of 92.7%. Thus, all 1019 HCC patients and 1138 controls were included in the final analysis.

Statistical analysis

Statistical analyses were performed using the SPSS 16.0 software (SPSS Inc., Chicago, IL, United States). Continuous variables were evaluated using the two-sample *t*-test. Categorical variables and genotype frequencies between the HCC patients and controls were compared using the Pearson's χ^2 and Fisher's exact test. Hardy-Weinberg equilibrium (HWE) was evaluated by a goodness-of-fit χ^2 test to compare the observed genotype frequencies with the expected ones. The association between SNP genotypes and HCC risk was estimated using unconditional logistic regression analysis and an odds ratio (OR) with 95% confidence interval (CI). All statistical tests were two-sided. A P-value < 0.05 was considered to indicate statistical significance.

RESULTS

Characteristics of the participants

The 2157 unrelated Chinese subjects enrolled in this study included 881 (86.5%) males and 138 (13.5%) females with HCC. The mean age of these patients was 48.54 ± 11.44 years. The control group consisted of 982 (86.3%) males and 156 (13.7%) females, with a mean age of 48.01 ± 11.5 years. The general demographic characteristics and behavior information on the patients and controls are provided in Table 2. There were no significant differences between the HCC patients and the controls in terms of age, sex, and nationality; however, HCC patients had a significantly higher rate of a positive history of HBV infection, a family history of HCC, smoking, and drinking.

Table 2 General demographic characteristics and behavioral information among hepatocellular carcinoma patients and controls

Variable	HCC patients *n* = 1019	Controls *n* = 1138	t/χ^2	*P* value
Age	48.54 ± 11.44	48.01 ± 11.50	-1.076	0.28
Gender				
Male	881	982	0.013	0.91
Female	138	156		
Nationality				
Han	673	708	3.591	0.17
Zhuang	332	410		
Others	14	20		
Drinking				
Yes	345	145	136.527	< 0.001
No	674	993		
Smoking				
Yes	355	158	130.222	< 0.001
No	664	980		
Chronic HBV infection				
Yes	794	109	1031.687	< 0.001
No	225	1029		
Family history of HCC				
Yes	80	2	86.597	< 0.001
No	939	1136		

HCC: Hepatocellular carcinoma.

Allele frequencies and genotype distribution

In the control group, the genotype frequencies of the 15 SNPs, all but *CDKN1A* rs3176320, were in line with the HWE ($P > 0.05$), which indicated that these study participants were from a homogeneous group. The allele frequencies and genotype distribution of SNPs among the HCC patients and controls from this study are listed in Table 3.

Association analysis of genetic polymorphisms and HCC

The association between SNPs and the risk of HCC was examined using unconditional logistic regression analysis. According to the crude ORs and their 95%CIs, *SMAD3* rs11556090 AG or AG/GG and *MCM7* rs2070215 GG carried an increased risk of HCC when compared with the wild genotype *SMAD3* rs11556090 AA and *MCM7* rs2070215 AA, respectively. Individuals with *CDC25C* rs3734166 GG or GA/GG and *KAT2B* rs4858770 TT had a lower risk of HCC than those with the wild genotype *CDC25C* rs3734166 AA and *KAT2B* rs4858770 CC, respectively. However, the association disappeared after adjusting for age, sex, nationality, smoking, drinking, family history of HCC, and HBV infection. Using individuals with the wild genotype AA as the reference, individuals carrying the GG variant of *KAT2B* rs17006625 had a higher risk of HCC (adjusted OR = 1.64, 95%CI: 1.01-2.64, $P = 0.04$) after adjusting for confounding factors. In addition, compared with the wild genotypes *MCM4* rs2305952 TT and *CHEK1* rs515255 CC, individuals carrying the CC variant of *MCM4* rs2305952 or the TC, TT, TC/TT variants of *CHEK1* rs515255 had a significantly lower risk of HCC (adjusted OR = 0.22, 95%CI: 0.08-0.63, $P = 0.01$; adjusted OR = 0.73, 95%CI: 0.56-0.96, $P = 0.02$; adjusted OR = 0.67, 95%CI: 0.46-0.97, $P = 0.04$; adjusted OR = 0.72, 95%CI: 0.56-0.92, $P = 0.01$, respectively). The associations are shown in Table 4.

Table 3 Allele frequencies and genotype distribution of single nucleotide polymorphisms *n* (%)

SNP	Genotype	HCC patients *n* = 1019	Control *n* = 1138	χ^2	*P* value of HWE
rs2305952	TT	801 (78.61)	883 (77.59)		
	TC	209 (20.51)	238 (20.91)	0.04	0.83
	CC	9 (0.88)	17 (1.49)		
rs2425675	GG	632 (62.02)	724 (63.62)		
	AG	348 (34.15)	374 (32.86)	0.96	0.33
	AA	39 (3.83)	40 (3.51)		
rs3088440	GG	750 (73.60)	813 (71.44)		
	GA	249 (24.44)	300 (26.36)	0.19	0.66
	AA	20 (1.96)	25 (2.20)		
rs3917148	AA	773 (75.86)	882 (77.50)		
	CA	233 (22.87)	235 (20.65)	1.32	0.25
	CC	13 (1.28)	21 (1.85)		
rs3929	GG	619 (60.75)	688 (60.46)		
	GC	349 (34.25)	395 (34.71)	0.03	0.86
	CC	51 (5.00)	55 (4.83)		
rs6987652	GG	743 (72.91)	843 (74.08)		
	AG	251 (24.63)	270 (23.73)	0.38	0.54
	AA	25 (2.45)	25 (2.20)		
rs11556090	AA	622 (61.04)	749 (65.82)		
	AG	352 (34.54)	346 (30.40)	0.15	0.70
	GG	45 (4.42)	43 (3.78)		
rs17006625	AA	526 (51.62)	620 (54.48)		
	AG	412 (40.43)	446 (39.19)	0.48	0.49
	GG	81 (7.95)	72 (6.33)		
rs2070215	AA	465 (45.63)	554 (48.68)		
	AG	424 (41.61)	480 (42.18)	< 0.01	1.00
	GG	130 (12.76)	104 (9.14)		
rs2261360	CC	460 (45.14)	484 (42.53)		
	CA	433 (42.49)	497 (43.67)	2.61	0.11
	AA	126 (12.37)	157 (13.80)		
rs3176320	AA	579 (56.82)	687 (60.37)		
	GA	383 (37.59)	377 (33.13)	5.05	0.02
	GG	57 (5.59)	74 (6.50)		
rs3734166	AA	421 (41.32)	421 (36.99)		
	GA	481 (47.20)	539 (47.36)	0.06	0.8
	GG	117 (11.48)	178 (15.64)		
rs4858770	CC	445 (43.67)	465 (40.86)		
	CT	461 (45.24)	515 (45.25)	0.65	0.42
	TT	113 (11.09)	158 (13.88)		
rs515255	CC	408 (40.04)	411 (36.12)		
	TC	469 (46.03)	553 (48.59)	0.29	0.59
	TT	142 (13.94)	174 (15.29)		
rs8025774	CC	313 (30.72)	335 (29.44)		
	CT	514 (50.44)	547 (48.07)	1.32	0.25
	TT	192 (18.84)	256 (22.50)		

HCC: Hepatocellular carcinoma; SNP: Single nucleotide polymorphism; HWE: Hardy-Weinberg equilibrium

Association between SNPs and HCC risk stratified by behavioral factors

HBV infection, alcohol intake status, and smoking status are important behavioral factors that can

Table 4 Associations between single nucleotide polymorphisms with the risk of hepatocellular carcinoma

SNP	Genotype	OR (95%CI)[1]	*P* value[1]	OR (95%CI)[2]	*P* value[2]
rs2305952	TT	Reference		Reference	
	TC	0.97 (0.79-1.19)	0.76	0.97 (0.72-1.32)	0.85
	CC	0.58 (0.26-1.32)	0.19	0.22 (0.08-0.63)	0.01[a]
	TC/CC	0.94 (0.77-1.16)	0.57	0.89 (0.66-1.19)	0.43
rs2425675	GG	Reference		Reference	
	AG	1.07 (0.89-1.28)	0.49	1.92 (0.71-1.20)	0.54
	AA	1.12 (0.71-1.76)	0.63	0.97 (0.51-1.85)	0.93
	AG/AA	1.07 (0.90-1.28)	0.44	0.93 (0.72-1.20)	0.56
rs3088440	GG	Reference		Reference	
	GA	0.90 (0.74-1.09)	0.29	1.02 (0.76-1.35)	0.92
	AA	0.87 (0.48-1.58)	0.64	1.46 (0.62-3.44)	0.38
	GA/AA	0.90 (0.74-1.09)	0.26	1.04 (0.79-1.37)	0.77
rs3917148	AA	Reference		Reference	
	CA	1.13 (0.92-1.39)	0.24	1.18 (0.88-1.59)	0.28
	CC	0.71 (0.35-1.42)	0.33	1.05 (0.41-2.68)	0.92
	CA/CC	1.10 (0.90-1.34)	0.37	1.17 (0.88-1.56)	0.29
rs3929	GG	Reference		Reference	
	GC	0.98 (0.82-1.18)	0.84	0.97 (0.75-1.26)	0.82
	CC	1.03 (0.69-1.53)	0.88	1.39 (0.80-2.42)	0.25
	GC/CC	0.99 (0.83-1.18)	0.89	1.02 (0.79-1.30)	0.90
rs6987652	GG	Reference		Reference	
	AG	1.06 (0.87-1.29)	0.60	0.92 (0.69-1.23)	0.59
	AA	1.14 (0.65-1.99)	0.66	1.26 (0.55-2.88)	0.59
	AG/AA	1.06 (0.88-1.29)	0.54	0.95 (0.72-1.25)	0.71
rs11556090	AA	Reference		Reference	
	AG	1.23 (1.02-1.47)	0.03	1.11 (0.85-1.44)	0.44
	GG	1.26 (0.82-1.94)	0.29	1.02 (0.54-1.91)	0.96
	AG/GG	1.23 (1.03-1.47)	0.02	1.10 (0.85-1.42)	0.47
rs17006625	AA	Reference		Reference	
	AG	1.09 (0.91-1.30)	0.35	1.07 (0.83-1.38)	0.61
	GG	1.33 (0.95-1.86)	0.10	1.64 (1.01-2.64)	0.04[a]
	AG/GG	1.12 (0.95-1.33)	0.18	1.14 (0.89-1.46)	0.29
rs2070215	AA	Reference		Reference	
	AG	1.05 (0.88-1.26)	0.58	0.95 (0.73-1.24)	0.71
	GG	1.49 (1.12-1.98)	0.01	1.39 (0.93-2.08)	0.11
	AG/GG	1.13 (0.95-1.34)	0.16	1.03 (0.81-1.32)	0.81
rs2261360	CC	Reference		Reference	
	CA	0.92 (0.77-1.10)	0.35	0.84 (0.64-1.09)	0.19
	AA	0.84 (0.65-1.10)	0.21	0.89 (0.60-1.31)	0.55
	CA/AA	0.90 (0.76-1.07)	0.22	0.85 (0.66-1.09)	0.19
rs3734166	AA	Reference		Reference	
	GA	0.89 (0.74-1.07)	0.22	0.92 (0.71-1.21)	0.56
	GG	0.66 (0.50-0.86)	0.002	0.86 (0.59-1.25)	0.43
	GA/GG	0.83 (0.70-0.99)	0.04	0.91 (0.71-1.17)	0.45
rs4858770	CC	Reference		Reference	
	CT	0.94 (0.78-1.12)	0.47	0.96 (0.74-1.24)	0.74
	TT	0.75 (0.57-0.98)	0.04	0.80 (0.54-1.20)	0.28
	CT/TT	0.89 (0.75-1.06)	0.19	0.92 (0.72-1.18)	0.51
rs515255	CC	Reference		Reference	
	TC	0.85 (0.71-1.03)	0.09	0.73 (0.56-0.96)	0.02[a]
	TT	0.82 (0.63-1.07)	0.14	0.67 (0.46-0.97)	0.04[a]
	TC/TT	0.85 (0.71-1.01)	0.06	0.72 (0.56-0.92)	0.01[a]
rs8025774	CC	Reference		Reference	
	CT	1.01 (0.83-1.22)	0.95	0.95 (0.72-1.27)	0.74
	TT	0.80 (0.63-1.02)	0.08	0.94 (0.66-1.32)	0.71
	CT/TT	0.94 (0.78-1.13)	0.52	0.95 (0.73-1.24)	0.69

[1]OR and 95%CI without adjusting for confounding factors; [2]OR and 95%CI after adjusting for age, sex, nationality, smoking, drinking, family history of hepatocellular carcinoma, and HBV infection. [a]$P < 0.05$ was considered statistically significant. OR: Odds ratio; CI: Confidence interval; SNPs: Single nucleotide polymorphisms.

increase the risk of HCC. To account for the role of these factors, a stratified analysis was conducted. Thus, when the patients were stratified, we found that the variant genotype CC of *MCM4* rs2305952 was associated with a significantly lower risk of HCC among HBsAg-positive individuals, non-drinkers, and non-smokers (adjusted OR = 0.25, 95%CI: 0.08-0.80, *P* = 0.02; adjusted OR = 0.19, 95%CI: 0.06-0.60, *P*

Table 5 Stratified analysis on the association between single nucleotide polymorphism genotype and hepatocellular carcinoma risk according to hepatitis B virus infection status

SNP	HBsAg-positive				HBsAg-negative			
	Case	Control	OR (95%CI)[1]	*P* value[1]	Case	Control	OR (95%CI)[1]	*P* value[1]
rs2305952								
TT	624	80	Reference		177	803	Reference	
TC	161	24	0.86 (0.53-1.42)	0.56	48	214	1.05 (0.72-1.52)	0.80
CC	9	5	0.25 (0.08-0.80)	0.02[a]	0	12	-	1.00
TC/CC	170	29	0.76 (0.48-1.21)	0.25	48	226	0.99 (0.68-1.43)	0.95
rs17006625								
AA	411	60	Reference		115	560	Reference	
AG	323	42	1.15 (0.75-1.76)	0.54	89	404	1.07 (0.77-1.48)	0.68
GG	60	7	1.36 (0.59-3.17)	0.47	21	65	1.79 (1.02-3.12)	0.04[a]
AG/GG	383	49	1.18 (0.78-1.77)	0.44	110	469	1.17 (0.86-1.59)	0.32
rs515255								
CC	301	39	Reference		107	372	Reference	
TC	377	52	0.93 (0.59-1.46)	0.75	92	501	0.64 (0.46-0.89)	0.01[a]
TT	116	18	0.81 (0.44-1.50)	0.51	26	156	0.69 (0.36-0.96)	0.03[a]
TC/TT	493	70	0.90 (0.59-1.37)	0.62	118	657	0.63 (0.46-0.86)	0.003[a]

[1]OR and 95%CI after adjusting for age, sex, nationality, smoking, drinking and family history of hepatocellular carcinoma. [a]$P < 0.05$ was considered statistically significant. OR: Odds ratio; CI: Confidence interval; SNPs: Single nucleotide polymorphisms.

Table 6 Stratified analysis on the association between single nucleotide polymorphism genotype and hepatocellular carcinoma risk according to drinking status

SNP	Drinking				Non-drinking			
	Case	Control	OR (95%CI)[1]	*P* value[1]	Case	Control	OR (95%CI)[1]	*P* value[1]
rs2305952								
TT	273	111	Reference		528	772	Reference	
TC	69	33	0.82 (0.44-1.52)	0.53	140	205	1.02 (0.72-1.44)	0.93
CC	3	1	0.51 (0.03-9.74)	0.66	6	16	0.19 (0.06-0.60)	0.004[a]
TC/CC	72	34	0.81 (0.44-1.49)	0.49	146	221	0.91 (0.65-1.27)	0.57
rs515255								
CC	145	56	Reference		263	355	Reference	
TC	154	71	0.69 (0.40-1.19)	0.18	315	482	0.73 (0.54-0.99)	0.05[a]
TT	46	18	1.10 (0.50-2.43)	0.82	96	156	0.56 (0.36-0.86)	0.01[a]
TC/TT	200	89	0.77 (0.46-1.29)	0.31	411	638	0.69 (0.52-0.92)	0.01[a]

[1]OR and 95%CI after adjusting for age, sex, nationality, smoking, family history of hepatocellular carcinoma, and hepatitis B virus infection. [a]$P < 0.05$ was considered statistically significant. OR: Odds ratio; CI: Confidence interval; SNPs: Single nucleotide polymorphisms.

= 0.004; adjusted OR = 0.17, 95%CI: 0.05-0.56, *P* = 0.004, respectively). The variant genotypes TC, TT, and TC/TT of *CHEK1* rs515255 were associated with a significantly lower risk of HCC in HBsAg-negative individuals (adjusted OR = 0.64, 95%CI: 0.46-0.89, *P* = 0.01; adjusted OR = 0.69, 95%CI: 0.36-0.96, *P* = 0.03; adjusted OR = 0.63, 95%CI: 0.46-0.86, *P* = 0.003) and in non-drinkers (adjusted OR = 0.73, 95%CI: 0.54-0.99, *P* = 0.05; adjusted OR = 0.56, 95%CI: 0.36-0.86, *P* = 0.01; adjusted OR = 0.69, 95%CI: 0.52-0.92, *P* = 0.01, respectively). Among smokers, those with the TC variant genotype of *CHEK1* rs515255 had a significantly lower risk of HCC (adjusted OR = 0.54, 95%CI: 0.32-0.93, *P* = 0.03), while among non-smokers the risk was significantly lower in those with the TT variant genotype (adjusted OR = 0.60, 95%CI: 0.39-0.94, *P* = 0.03). In addition, the variant genotype GG of *KAT2B* rs17006625 was shown to carry a significantly higher risk of HCC among HBsAg-negative individuals (adjusted OR = 1.79, 95%CI: 1.02-3.12, *P* = 0.04). These findings are summarized in Tables 5-7 (only significant SNPs are shown).

DISCUSSION

We performed this case-control study to investigate the associations between the 15 SNPs in 12 cell cycle pathway genes and the risk of HCC. The *KAT2B* rs17006625 GG was associated with an increased risk of HCC. Furthermore, this harmful effect was more marked in HBsAg-negative carriers. Conversely, the *CHEK1* rs515255 TC, TT, TC/TT and the *MCM4* rs2305952 CC were associated with a decreased risk of HCC. In addition, the risk was markedly lower for those who were carriers of *MCM4* rs2305952 CC and were also HBsAg-positive and non-drinking and non-smoking and for those who were carriers of the TC,

Table 7 Stratified analysis on the association between single nucleotide polymorphism genotype and hepatocellular carcinoma risk according to smoking status

SNP	Smoking				Non-smoking			
	Case	Control	OR (95%CI)[1]	*P* value[1]	Case	Control	OR (95%CI)[1]	*P* value[1]
rs2305952								
TT	274	124	Reference		527	759	Reference	
TC	77	32	1.05 (0.58-1.91)	0.87	132	206	0.94 (0.66-1.34)	0.75
CC	4	2	0.54 (0.06-4.97)	0.59	5	15	0.17 (0.05-0.56)	0.004[a]
TC/CC	81	34	1.01 (0.57-1.82)	0.96	137	221	0.84 (0.60-1.19)	0.33
rs515255								
CC	145	53	Reference		263	358	Reference	
TC	155	84	0.54 (0.32-0.93)	0.03[a]	314	469	0.81 (0.59-1.10)	0.17
TT	55	21	0.87 (0.41-1.85)	0.72	87	153	0.60 (0.39-0.94)	0.03[a]
TC/TT	210	105	0.61 (0.67-1.02)	0.06	401	622	0.75 (0.56-1.01)	0.06

[1]OR and 95%CI after adjusting for age, sex, nationality, drinking, family history of hepatocellular carcinoma, and HBV infection. [a]$P < 0.05$ was considered statistically significant. OR: Odds ratio; CI: Confidence interval; SNPs: Single nucleotide polymorphisms.

TT, TC/TT genotype of *CHEK1* rs515255 and were also HBsAg-negative and non-drinking. No significant associations were observed between other 12 SNPs and HCC risk.

The cell cycle pathway is one of the most important cellular signaling pathways, as it regulates both cell division and apoptosis. DNA damage readily leads to dysregulation of the cell cycle, which is an essential step in the initiation and development of human malignancies[21-23]. In the present study, we reported that three SNPs in cell cycle pathway genes (*MCM4*, *CHEK1*, and *KAT2B*) were significantly associated with the risk of HCC.

MCM4, a member of the mini-chromosome maintenance family of proteins, which interact with cell cycle checkpoints and recombinant proteins to stabilize the S phase, is essential for the initiation of eukaryotic genome replication[24,25]. Several reports have shown that MCM4 protein is overexpressed in esophageal carcinomas[26], cervical cancer[27], and cervical squamous cell carcinoma[28]. In our study, we found that the polymorphism of *MCM4* rs2305952 was associated with a lower risk of HCC. However, the mechanism of *MCM4* polymorphisms in HCC development remains unclear. Ishimi *et al*[29] found that MCM4 is one of the crucial targets of DNA replication checkpoint and the phosphorylation of MCM4, which is caused by the activation of ATR-CHK1 pathway and CDK2, results in the DNA replication through the inactivation of the MCM4/6/7 complex. It is also found that *MCM4* mutations may cause tumors by affecting the formation of the MCM4/6/7 complex[30,31].

CHEK1 is a mediator of cell cycle arrest in response to DNA damage. In addition to controlling cell cycle progression[32], it regulates DNA repair[33] and coordinates cell survival and death[34,35]. It is reported that CHEK1 plays an important role in the checkpoint of DNA damage and DNA replication through the ATR-CHK1 pathway[36-38]. Lin *et al*[39] performed a meta-analysis to explore the association of *CHEK1* SNPs with breast cancer in patients registered in the database of the Utah Breast Cancer Study. They found that *CHEK1* polymorphisms are significantly associated with the risk of breast cancer. However, in that study common alleles of *CHEK1* are not implicated in breast cancer risk or in the survival of breast cancer patients after meta-analysis. Our results showed an association between the *CHEK1* rs515255 genetic variant and a decreased risk of HCC, after adjusting for age, sex, nationality, smoking, drinking, family history of HCC, and HBV infection. The conflicting results may reflect the different cancers evaluated and/or differences in the study population. This remains to be clarified in further investigations.

KAT2B, also known as *PCAF*, encodes the cofactor PCAF (P300/CBP associated factor) of activated nucleoprotein that is important in cell cycle regulation. KAT2B induces cell cycle arrest and/or apoptosis by regulating p53 and affects the acetylation and stability of E2F1 in the presence of DNA damage[40,41]. Overexpression of PCAF was reported in samples of both central nervous system tumors and Wilm's tumors[42]. In addition, an association between *KAT2B* gene polymorphisms and several human diseases and behaviors has been reported. For example, the *KAT2B* SNP rs9829896 is associated with drug abuse in African Americans[43]. We also found that the risk of HCC was higher in individuals with the *KAT2B* rs17006625 GG genotype than with the AA genotype, after adjusting for age, sex, nationality, smoking, drinking, family history of HCC, and HBV infection.

HBV infection status, drinking status, and smoking status are well known to influence the occurrence and development of HCC[44-47]. Moreover, some genotypes have no effect on HCC risk when considered within a population as a whole, but the subgroup analysis may show an effect on HCC risk among alcohol drinkers and/or smokers[48,49]. Therefore, in our study, we evaluated the role of risk factors such as drinking status and smoking status in a stratified analysis and

found that these environmental factors may interact with the analyzed SNPs.

Our study had several limitations. First, the research population was drawn only from the Guangxi Zhuang Autonomous Region. Whether the results apply to the Chinese population as a whole or to other ethnic groups remains to be seen. Second, because our study used a case-control format, recall bias was difficult to avoid. However, we sought to minimize recall bias by choosing patients newly diagnosed with HCC. Finally, the functional influence of the examined SNPs and the potential mechanisms need to be determined in functional validation tests.

In conclusion, *MCM4* rs2305952 CC and *CHEK1* rs515255 TC, TT, TC/TT may decrease the risk of HCC and *KAT2B* rs17006625 GG may increase the risk of HCC. In addition, we observed an increased risk associated with *KAT2B* rs17006625 GG in HBsAg-negative patients. Furthermore, we also observed a decreased risk associated with *MCM4* rs2305952 CC in HBsAg-positive patients and in also non-drinking patients and non-smoking patients, and with *CHEK1* rs515255 TC, TT, TC/TT in HBsAg-negative patients and in also non-drinking patients. Our results suggest that the genetic variants in the cell cycle pathway genes affect the risk of HCC, however, further studies are needed to confirm the findings.

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COMMENTS

Background

The uncontrollable proliferation of cancer cells is a crucial mechanism in cancer development and progression. Previous studies have shown that polymorphisms of cell cycle pathway genes are associated with cancer. However, their relationship with hepatocellular carcinoma (HCC) is unclear.

Research frontiers

Despite reports of an association between polymorphisms in cell cycle pathway genes and cancer risk, little is known about the relationship between these polymorphisms and HCC risk.

Innovations and breakthroughs

This study enrolled 1127 cases newly diagnosed with HCC and 1200 non-tumor patients. It comprehensively investigated the relationship between 15 SNPs in 12 cell cycle pathway genes and HCC risk.

Applications

Since individuals with the *KAT2B* rs17006625 GG genotype may have an increased risk of HCC, they should be carefully monitored to reduce the occurrence and development of HCC.

Terminology

A single nucleotide polymorphism (SNP) is a variation in the genomic DNA sequence. SNPs in some genes may cause an increased or decreased risk of HCC.

Peer-review

The manuscript is interesting and provides relevant information. The study is a descriptive paper analyzing the polymorphism in HCC in a wide number of patients. The analyses are consistent with the results and the conclusions asserted in the manuscript.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Hepatitis E in Israel: A nation-wide retrospective study

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Abstract

AIM: To investigate the epidemiology, risk factors and clinical course of acute hepatitis E virus (HEV) infection in Israel, an industrialized country.

METHODS: A retrospective analysis of acute HEV cases diagnosed in Israel from 1993 to 2013. Acute HEV was defined by ALT/AST elevation and a positive HEV PCR test or positive anti-HEV-IgM serology. HEV RNA was tested by quantitative reverse transcription PCR. Antibodies to HEV were tested retrospectively using an ELISA assay. HEV-RNA was sequenced using RT-PCR of ORF1 and ORF2 regions to diagnose genotype of the virus. Epidemiologic and clinical data were collected by reviewing the clinical files and through a telephone interview according to a structured questionnaire.

RESULTS: Acute HEV was diagnosed in 68 patients. Among the 59 patients who gave an informed consent and were interviewed, 41% of infections were autochthonous (acquired in Israel), 44% travel-related and 15% imported by foreign workers. Autochthonous patients were mainly females (62.5%), more than half of them pregnant, 26% recalled consuming food or water in areas with poor sanitation, 44% ate non-kosher meat. Fulminant hepatitis developed in 3 patients (5%), all of them were females, two of them with post-partum infection, all acquired the disease in Israel (autochthonous). Israeli travelers with imported infection were predominantly males (73%), acquired the disease in the Indian subcontinent (81%), with 100% reporting having consumed fresh vegetables and drinks with ice cubes abroad. Six patients' sera were tested for genotype and revealed HEV genotype 1 (all cases acquired in the Indian subcontinent).

CONCLUSION: This is the first report which highlights the existence of hepatitis E as an autochthonous infection in Israel. Imported HEV originates mostly from the Indian subcontinent.

Key words: Hepatitis E; Autochthonous; Travel; Foreign workers; Pregnancy; Post-partum; India; Nepal; Indian subcontinent; Israel

Core tip: This is the first epidemiologic report on hepatitis E virus (HEV) in Israel. This report demonstrates the significant presence of autochthonous acute HEV in Israel, serving as an example of occurrence in an industrialized country. Suspected risk factors in Israel include consumption of water and food in areas with poor sanitation, exposure to animals and eating a non-Kosher meat. The high risk group for fulminant hepatitis was pregnant women in their final trimester. Additionally, imported HEV, originating mainly from the Indian subcontinent, is also seen in Israel. Awareness of this disease is important both among physicians in Israel as well as those in other industrialized countries.

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INTRODUCTION

Hepatitis E virus (HEV) infection is currently one of the leading causes of viral hepatitis worldwide[1]. The first epidemic of HEV infection was recognized in India, by a retrospective epidemiologic and serologic survey performed in India and the United States in the early 1980s. This led to the recognition of HEV as a water-borne associated hepatitis. Similar epidemics were subsequently identified in Central and Southeast Asia, the Middle East and North Africa[2-4]. The viral genome was later cloned and sequenced using samples of bile obtained from experimentally infected macaques, and the virus was named Hepatitis E[5-8].

HEV is a single strand RNA virus classified in the genus Hepevirus, family Hepeviridae[9]. Identification of four HEV genotypes has subsequently been used to study the molecular epidemiology of HEV infection worldwide. Genotypes 1 and 2, restricted to humans, are mostly seen in developing countries causing large waterborne outbreaks of hepatitis. Genotype 1 is mostly associated with outbreaks in Asia and Africa, whereas genotype 2 has been detected in Mexico and some African countries[10]. In recent years there has been increasing evidence of an autochthonous HEV infection in industrialized countries, contrasting with previous reports of HEV as only an imported infection from endemic, developing countries. In these cases, genotypes 3 and 4 have been identified, and found to be responsible for sporadic cases of autochthonous hepatitis E in industrialized countries[11-15]. In contrast to genotypes 1 and 2, genotypes 3 and 4 infect not only humans but also other mammalian species such as pigs, boars, and deer[11].

HEV is transmitted predominantly *via* the fecal-oral route, causing a self-limiting disease which resolves spontaneously within 4-6 wk[6]. Occasionally, in immune-suppressed patients and in pregnant women, a fulminant form of hepatitis develops[16]. Chronic infection has been identified almost exclusively among immunocompromised persons, including organ-transplant recipients, patients receiving cancer chemotherapy, and HIV-infected persons[17].

Israel is an industrialized country located amid HEV endemic countries and home to immigrants and refugees from African countries (such as Egypt, Sudan and Ethiopia, all endemic for genotype 1 of the virus). Furthermore, since a portion of Israel's population eats only kosher food (*i.e.,* avoiding pork, game meat or seafood), the index of suspicion for autochthonous cases has been low. Data obtained through old and non-validated immune-assays regarding sero-epidemiology of hepatitis E in Israel revealed a seroprevalence of 2.81% and 1.81% in the Jewish and Arab population, respectively[18]. However, data regarding acute HEV revealed only one case report of acute HEV infection acquired in Israel[19] while the remaining published cases were travel-related[20,21].

The aim of this study was to identify whether there is a change in the epidemiology of acute HEV in Israel, with cases acquired in Israel (autochthonous cases) and to characterize the epidemiology, risk factors and clinical presentation of all documented acute HEV infections in patients diagnosed in Israel.

MATERIALS AND METHODS

Study design

A descriptive, retrospective, nation-wide study.

Patient population

The study included all patients diagnosed with acute HEV infection in Israel from October 1993 to 2013 at the laboratory of the Liver Unit at the Hadassah Medical Center in Jerusalem. During the study period, this laboratory was the only reference laboratory in Israel for HEV detection. Epidemiologic and clinical data were collected by reviewing the clinical files and through telephone interviews in accordance with a structured questionnaire. The study was approved by the Sheba-Medical Centers' institutional review board.

Case definitions

"*Definite acute HEV*" was defined as acute hepatitis manifested by ALT/AST elevation and a positive HEV PCR (polymerase chain reaction) test or positive anti-HEV-IgM serology. "*Probable acute HEV*" was defined as acute non-A, non-B, non-C hepatitis with negative HEV PCR and negative anti-HEV-IgM serology but positive for anti-HEV-IgG serology, where serum samples were taken later in the course of the disease, and with a clinical course that fit HEV infection, and no other proven etiology. "*Fulminant hepatitis*" was defined as a rapid development of acute liver injury with evidence of coagulation abnormality, an international normalized ratio (INR) > 1.5, and any degree of hepatic encephalopathy in a patient without pre-existing liver disease; and after exclusion of the conventional etiologies for acute liver failure[22].

Laboratory tests

Serologic detection of anti-HEV antibodies: Antibodies to HEV (IgG and IgM) were tested using an ELISA micro-titer plates assay [DS-EIA-anti-HEV-G, DS-EIA-anti-HEV-M, DSI S.R.L. Serronno (VA), Milan, Italy] according to the manufacturer's instructions. Micro-titer plates were coated with HEV peptides able to detect all four genotypes of HEV. Ten μL serum samples were diluted 1:10 with 90 μL diluent and incubated for 30 min at 37 ℃ and rinsed three times, followed by a second incubation with 100 μL conjugated antibodies for another 30 min at 37 ℃. After rinsing three times, 100 μL substrate was incubated for 30 min at room temperature. Finally, 100 μL stop solution was added followed by reading the plates at a 450 nm wave length. Sample reading > 0.2 OD were considered as positive.

A pangenotypic evaluation by CDC of 6 serologic assays for IgM against HEV identified the assay manufactured by diagnostic systems, which was used in this study, as having the best performance characteristics. Its diagnostic sensitivity and specificity were 98% and 95.2%, respectively[23].

We used an assay for the detection of IgG against HEV from the same manufacturer, with a sensitivity of 100% and specificity of 97.5%[24].

Detection of HEV-RNA by Taqman real time PCR: Detection of HEV RNA was performed by quantitative reverse transcription PCR (qRT-PCR)[25]. RNA was extracted from 200 μL serum with TRI reagent (Bio Lab), then diluted in 10 μL DEPC water. A 10 μL RNA aliquot was used for one step RT-PCR assay in a final volume of 20 μL. The region used for the real time assay is a highly conserved region, junction of ORF's 2/3 of HEV: HEV Forward primer GGTGGTTTCTGGGGTGAC, HEV Reverse primer AGGGGTTGGTTGGATGAA, HEV Probe FAM-TGATTCTCAGCCCTTCGC-BHQ.

HEV sequence: To define HEV genotype in the sera samples, we ran reverse transcription polymerase chain reaction (RT-PCR) of two regions of the virus: ORF1 and ORF2. PCR products were sent for cleaning and sequencing in a service laboratory (HY-lab). We used the programs CHROMAS and CLUSTAL in order to analyze the sequences.

Statistical analysis

Quantitative variables are presented as mean ± SD or as medians and range. Qualitative variables are presented as frequencies and ratios (percent). The χ^2 and the Fisher's exact tests were applied to assess associations between two qualitative variables. The comparison of quantitative variables between two independent groups was carried out using the two-sample *t*-test or the non-parametric Mann-Whitney test. All tests applied were two-tailed, and a *P*-value of 5% or less was considered statistically significant.

RESULTS

During the years 1993 to 2013, 651 patients with presumed acute non-A, non-B, non-C hepatitis were tested for HEV in Israel. Acute HEV was diagnosed in 68/651 patients (10.45%). Among them, 61 patients (90%) were classified as having "definite acute HEV" confirmed by a positive HEV-RNA PCR result (n = 50) or positive anti-HEV-IgM serology (n = 10). One patient had a positive PCR result from a stool sample taken abroad. Probable acute HEV was diagnosed in 7 patients.

Altogether the cohort of acute HEV infection included 68 patients, 58.8% male, with a mean age of 39.4 years. The greatest number of patients were between the ages of 17-40 years (63.5%). Comparing acute HEV positive patients with non-A-non-B-non-C-non-E acute hepatitis patients revealed no significant differences in gender or age distribution (Table 1).

Among this cohort of 68 patients with a history of acute HEV, 59 patients gave an informed consent and

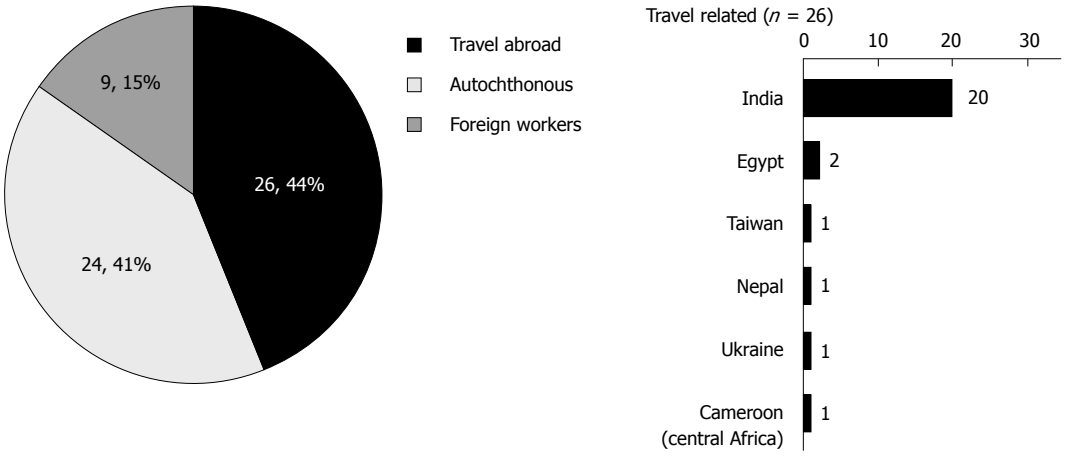

Figure 1 Hepatitis E infection in Israel according to place of acquisition (*n* = 59). Foreign workers origin: Nepal (*n* = 5), China (*n* = 1), 3 unknown.

Table 1 Demographic characteristics of patients with acute hepatitis E *vs* non A-B-C-E acute hepatitis

	Acute hepatitis	
	Non A-B-C-E hepatitis[1] *n* = 583	Hepatitis E[1] *n* = 68
Gender		
M	51.0%	58.8%
F	49.0%	41.1%
Age (yr)		
0-16	29 (7.0)	1 (1.6)
17-40	206 (49.8)	40 (63.5)
41-60	132 (31.9)	13 (20.6)
> 60	47 (11.4)	9 (14.3)
Mean	38.96	39.38
Range	0-86	16-87

[1]Comparing the groups above in parameters of gender and age reveled non-significant *P*-value.

were further evaluated. Thirty five patients (35/59, 59%) had "imported" HEV; among them 26 cases (26/59, 44%) were travel-related HEV infections in Israeli patients and 9 cases (9/59, 15%) were diagnosed in foreign workers from HEV endemic countries (Figure 1). The majority (80%) of travel-related HEV cases were acquired in the Indian subcontinent. Finally, 41% of the patients (24/59) did not travel abroad and had no contact with people from endemic areas, and are therefore defined as "autochthonous HEV".

There was a trend of an increasing number of cases diagnosed with acute hepatitis E throughout the study years in both the travel-related and autochthonous groups (Figure 2).

Autochthonous HE

This group consisted of 24 patients, predominantly female (15/24, 62.5%), with a mean age of 42 years old (SD-15, range: 15-69 years old) and without any contact with a foreign worker in Israel. There were, however, 26% (5/19) who recalled consuming food or water from rural settlements and areas of low sanitation (the West Bank, Bedouin villages) during the 6 wk before the onset of symptoms. Other probable risk factors for HEV infection are summarized in Table 2; 44%, (8/18) ate non-kosher meat (14% ate raw meat, 10% consumed sea food); 40% (8/20) reported contact with animals (cats, dogs, chicken, parrots, geese, fish, guinea pigs, horses or a monkey). Five out of the 24 with autochthonous infections (21%) had chronic liver disease before acquiring HEV (chronic HCV, HBsAg carrier, cystic fibrosis of liver or autoimmune hepatitis). Four of them were diagnosed by positive molecular test (PCR), and one by positive anti-IgM serology for HEV. Eight percent (2/24) received immune suppressing medications (Corticosteroids, Azathioprine, Mycophenolate Moftil and Tacrolimus). Among the female patients, 53% (8/15) were pregnant or post-partum at the time of clinical presentation.

Fifteen of the patients with autochthonous HEV infection (15/24, 62.5%) were diagnosed by detection of HEV-RNA in their serum. Unfortunately, we were unable to retrospectively test for the genotype in this group of autochthonous patients due to a breakdown in refrigeration.

Travel related HEV

This group consisted 26 patients, predominantly male (19/26, 73%), with a mean age of 37.38 years, SD-16.7. Among the females, 1 out of 7 was pregnant (14.3%). Sixty four percent (16/25) developed symptoms after returning to Israel, with a mean time elapsing before symptoms of 16 d (range: 2-28 d). Duration of travel was on average 62.5 d (range: 3-240 d). Thirty six percent (9/25) were symptomatic before flying back to Israel, and among those patients, the average duration of travel was 6.5 mo (range: 1-24 mo).

Behavior during travel, possibly contributing to risk of infection is summarized in Table 3. As described, the vast majority had contact with suspected contaminated water and raw vegetables. The entire group consisted

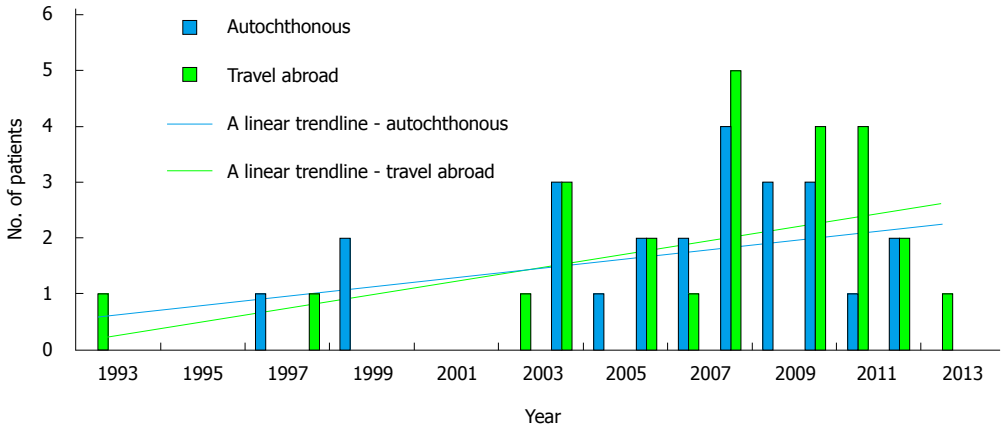

Figure 2 Number of cases of acute hepatitis E virus infection in travel related and autochthonous cases.

Table 2 Clinical characteristics and potential risk factors for hepatitis E virus in patients with autochthonous and travel-related hepatitis E virus infection[1]

Character		Autochthonous infection (n = 24)	Travel related (n = 26)	P value[2]
Demography	Gender: M	9 (37.5)	19 (73.1)	0.011
	Age: mean	41.58	37.38	0.358
	Range	15-69	20-74	
Potential risk factors: food related	Eating non-kosher meat	8 (44.4)	22 (81.8)	0.014
	Eating raw meat	3 (14.3)	3 (13.0)	1.000
	Eating sea-food	2 (10.0)	6 (28.6)	0.238
	Consuming food/water from areas with poor sanitation	5 (26.3)	2 (12.5)	0.415
	Contact with animals[3]	8 (40.0)	8 (34.8)	0.724
Potential risk factors: others	Pregnancy	8 (53.3)	1 (14.3)	0.165
	Immunosuppression[4]	2 (8.3)	0 (0.0)	0.225
	Chronic liver disease	5 (20.8)	1 (3.8)	0.064
Clinical data	Time from onset of symptoms to diagnosis (d)	59.13 (n = 15)	25.21 (n = 19)	0.009
	Duration of symptoms (average weeks)	5.94 (n = 18)	4.08 (n = 20)	0.149
	Hospitalization (percent of patients)	68.2% (15/22)	20 (80.0)	0.345
	Duration of hospitalization (d)	22.15 (n = 13)	11.11 (n = 19)	0.195
Laboratory tests (average)	Bilirubin mg/dL (STD)	10.95 (10.84)	9.24 (5.93)	0.813
	GPT (ALT) U/L (STD)	1169.3 (1279.4)	2446.4 (1604.3)	0.043
	GOT U/L(STD)	1311.7 (2114.6)	1540.4 (1412.7)	0.436
	ALKP (STD)	566.5 (986.1)	205.6 (54.2)	0.673
	GGT U/L (STD)	470.0 (625.1)	232.2 (243.3)	0.730
	LDH U/L (STD)	2613 (6400.5)	1503 (1511.4)	0.440
	ALB g/dL (STD)	3.3 (0.94)	3.9 (0.42)	0.241
	INR (STD)	1.42 (0.8)	1.21 (0.2)	0.791
Outcome	Self-limited	20 (86.9)	26 (100)	0.085
	Fulminant hepatitis	3 (13)	0 (0)	
	Chronic hepatitis	0 (0)	0 (0)	

[1]Excluding foreign workers with acute HEV; [2]Mann-Whitney *U* Test, the χ^2 and the Fisher's exact tests were applied as detailed in the text (Methods); [3]Contact with animals *i.e.,*: cats, dogs, chicken, parrots, goose, fish, guinea pigs, horses, and monkeys; [4]Immune-suppressed patients: (1) Forty five years old woman treated with immune-suppressed therapy (Imuran) for autoimmune hepatitis; and (2) 19 years old woman treated with immune suppressed therapy after liver and kidney transplantation due to cystic fibrosis.

of healthy travelers, none took immunosuppressive drugs or had a known systemic disease associated with immunosuppression, with the exception of one patient who had chronic hepatitis B, diagnosed in the past. Foreign workers from endemic countries (n = 9) were not included in the travel-related HEV group.

Twenty of the patients with travel-related HEV (20/26, 77%) were diagnosed by detection of HEV-RNA in their serum. Among them, we were able to sequence the HEV genome in six of the patients with available sera. All cases revealed genotype 1.

Comparing patients with travel-related infection and autochthonous infection revealed that the autochthonous patients were predominantly female (62.5% *vs* 27%, $P < 0.05$), had lower levels of alanine aminotransferase on presentation (mean 1169 U/L *vs* 2446 U/L, $P < 0.05$) and the time from onset of symptoms to diagnosis was more than twice as long (59 d *vs* 25 d; $P < 0.01$) (Table 2). Although there was no significant statistical difference in outcome between the two

Table 3 Potential risk factors for travel-related hepatitis E virus infection

Risk factor	No. of patients (total: n = 26)	Incidence
Gender - M	19/26	73.07%
Pregnancy	1/7	14.28%
Chronic liver disease[1]	1/23	4.30%
Immunosuppression	0/23	0.00%
Eating non-kosher meat	18/22	81.81%
Eating raw meat	3/23	13.04%
Eating sea-food	6/21	28.57%
Drinking tap water abroad	8/23	34.78%
Consuming drinks with ice cubes	23/23	100.00%
Brushing teeth with tap water	20/23	86.95%
Eating fresh vegetables abroad	23/23	100.00%
Bath in fresh water	15/23	65.21%
Contact with animals[2]	8/23	34.78%
Contact with travelers having similar symptoms	2/25	8.00%

[1]Chronic hepatitis B virus; [2]Contact with animals *i.e.*,: cat, dog, chicken, parrot, goose, fish, guinea pig, horse and monkey.

groups (due to small sample size), the results may indicate that patients with autochthonous HEV are more prone to fulminant hepatitis than patients with travel-related HEV infection (13% *vs* 0% respectively). There were no significant differences in age, hospitalization rate and symptom duration between the groups.

Pregnant women

In this study, there were nine cases of HEV in pregnant women (9/28 women in the study, 32%). Only one of the women was a returning traveler from India, while the rest were diagnosed with autochthonous infection (8/9, 88.9%). Fulminant hepatitis occurred in two of the pregnant women (2/9, 22%), without fatality or vertical transmission. A detailed description of this cohort, together with other cases of acute HEV in pregnant women from Western countries, was published recently[26].

In the entire cohort of the Israeli patients, the most common signs and symptoms were fatigue and non-obstructive-jaundice in 84% (26/31) and 78% (40/51) of patients respectively; 61% (11/18) of patients developed abdominal pain and 41% (19/46) had fever (> 37.5 ℃). Other complaints were nausea (40%, 19/48), diarrhea (21%, 10/48) and headache (24%, 11/45). Nine percent (4/43), developed neurologic abnormalities including epilepsy, encephalopathy or loss of consciousness. Admission to hospital in Israel was reported by 78% of patients, for a mean duration of 15.6 d (range: 1-84 d, SD-19.4). One Israeli traveler was admitted to an Indian hospital while abroad where HEV-RNA was identified in a stool sample by PCR.

Results of conventional liver tests and INR at presentation or later were retrieved in only 22 patients (Table 2). The rate of bilirubin level varied between 0.39 to 27.9 mg/dL. No clinically significant bleeding disorders were reported although coagulopathy was recorded in 41% of the patients (7/17), who presented with prolonged INR up to 3.49. Hepatocellular injury manifested in ALT elevation was significantly higher in patients with travel-related hepatitis compared to autochthonous infection (mean 2246.4 U/dL *vs* 1169.3 U/dL respectively, $P < 0.05$).

Ninety four percent (46/49) of the patients with HEV infection had self-limiting disease (Table 2). Among the travelers, none of the patients had a chronic or fulminant course of infection. This included a pregnant woman with HEV infection in her third trimester, who had a self-limiting infection, without any pregnancy or fetal related complications. In contrast, among the patients with autochthonous infection, three patients (3/23, 13%) developed fulminant hepatitis. All three were females in their reproductive years, two of them post-partum and the third had cystic fibrosis.

DISCUSSION

The current study was designed to review retrospectively the epidemiology and clinical outcome of acute HEV cases diagnosed in Israel in the past 20 years. During this period, 68 patients were diagnosed with acute HEV infection, and from those patients, 59 gave their consent for evaluation. In this cohort, 44% were travel related, 15% affected foreign workers and surprisingly 41% were autochthonous infection. This is the first study which recognizes the existence of a relatively large cohort of autochthonous HEV infections in Israel, while previous reports suggest that almost all cases of hepatitis E were travel related as summarized in Table 4.

Recently, reports on autochthonous HE in industrialized countries have become more frequent. The distribution of autochthonous *vs* travel-related cases varies widely between different countries[13,14,27-30], as can be seen in Table 5. In Israel about half of the cases are autochthonous.

As shown in this report (Figure 2), the number of HEV cases increased during the study years in both travelers and the autochthonous groups. This may reflect an increase in infection rates, but may also be the consequence of improved diagnostic tools and growing awareness among physicians in Israel of HEV infection.

Previous studies in industrialized countries linked sporadic cases of HEV to consumption of undercooked pork[1,12,31], raw game meat, shellfish[32] and to blood transfusions[33,34]. In autochthonous HEV in Israel, the most frequent risk factors included eating non-kosher meat and being exposed to animals. Furthermore, about a quarter of the patients reported that during the 6 wk that preceded the symptoms, they consumed food and/or water from the West Bank or other rural areas in Israel known to have relatively lower hygienic standards as compared to the rest of the country.

Table 4 Summary of the published case reports of acute hepatitis E virus in Israeli patients

Ref.	Year of follow-up	No. of patients	Travel related/autochthonous	Diagnosis
Schwartz *et al*[20]	1992-1998	5	Travel related (all cases acquired in the Indian subcontinent)	Serology tests (Abbott Laboratories, Abbott Park, IL, United States)
Lachish *et al*[21]	1997-2012	19	Travel related (84% acquired in the Indian subcontinent)	Molecular or Serology (IgM/IgG) tests (EIA, Abbott Laboratories, Abbott Park, IL, United States)
Mechnik *et al*[19]	2001	1	Autochthonous	Molecular test (HEV-RNA pos. in serum sample)

HEV: Hepatitis E virus.

Table 5 Hepatitis E virus in industrialized countries[1]

Ref.	No. of patients	Travel related	Autochthonous
Norder *et al*[27]	248	88.3%	11.7%
Romanò *et al*[14]	134	81.3%	18.7%
Ramalingam *et al*[28]	16	62.5%	37.5%
Israel (current paper)	68	44.0%[2]	41.0%
Drobeniuc *et al*[29]	26	42.0%	58.0%
Chalupa *et al*[13]	49	3.90%	96.1%
Mansuy *et al*[30]	62	3.20%	96.8%

[1]A Literature review of the prevalence of HEV infection in industrialized countries[13,14,27-30]; [2]the rest 15% were foreign workers. HEV: Hepatitis E virus.

The other major risk group susceptible to HEV was the Israeli travelers (44% of acute HEV in Israel). In a prospective observational study from Israel, 1% of ill-returning Israeli travelers were diagnosed with acute hepatitis (1997-2012), while HEV has become the most common hepatitis, with a prevalence of 39%, of all hepatitis cases. In the aforementioned study, 84% of the travel-related HEV cases were "imported" from the Indian subcontinent[21]. In this study, 80.7% of travel associated cases were acquired in the Indian subcontinent. In travel-related HEV, in contrast to autochthonous HEV, the majority of patients were young adult males (73% male, average age of 37 years). Only a minority of patients had chronic liver disease (3.8%). Regarding risk behaviors during travel in this population, although the majority of patients followed pre-travel instructions and did not drink tap water abroad, all patients ate raw vegetables, used ice cubes, brushed their teeth with tap water and bathed in fresh water (Table 3). A control group of travelers without HEV was not available for this study.

The comparison of characteristics of autochthonous and travel-related acute HEV infection, revealed significant differences between the two groups (Table 2); the majority of infected travelers were male, while in the autochthonous group, the majority were female. Time to diagnose was significantly longer in the autochthonous group (on average, more than twice longer), suggesting a delay in diagnosis. ALT levels were significantly higher in infected travelers than in patients with autochthonous infection.

HEV is generally a self-limiting disease, although on rare occasions it can be fulminant[6,16]. In the present study, most of the patients with acute HEV infection had a self-limited disease. Patients who had fulminant hepatitis reported one of the following risk factors: pregnancy (including post-partum women), immune-suppression or chronic liver disease.

There is scarce evidence about the mortality of pregnant women with acute HEV infection in industrialized countries[35-38]. Our previous report indicated that in an industrialized country such as Israel, pregnant women had a high risk of fulminant hepatitis during their final trimester (2/9, 22.2%), though with no mortality or vertical transmission[26].

Patients with underlying chronic liver disease who develop hepatitis E have a poor prognosis, as they frequently develop acute or sub-acute liver failure[17]. In a study of a large cohort of patients in India with chronic liver disease, patients who developed HEV associated liver failure had a significantly worse prognosis than patients who decompensated due to other causes. In this cited cohort, the 12-mo mortality with HEV infection reached 70%[39]. In industrialized countries, smaller studies have also shown a poor prognosis for HEV infected patients with underlying chronic liver disease[40,41]. In our current study, evaluation of the entire group of 69 patients with acute HEV infection, revealed 7 patients with underlying chronic liver disease (chronic HCV, chronic HBV, cystic fibrotic liver, autoimmune hepatitis, idiopathic cirrhosis). Six of the patients survived and were interviewed and only one patient with chronic HBV infection and cirrhosis died 4 years after the diagnosis of chronic hepatitis B and 3 years after the diagnosis of acute HEV infection.

The differential diagnosis of autochthonous HEV infection includes drug induced liver injury (DILI). Two reports from the United Kingdom and the United States revealed that 21.4% and 3%, respectively, of the patients with an initial diagnosis of DILI, had autochthonous HEV. Such misdiagnosis is particularly common in elderly populations with autochthonous HEV taking various potentially hepato-toxic medications and herbs[42,43]. In the present cohort, two patients treated with Isoniazide and Ketoconazole who presented with acute hepatitis, were initially misdiagnosed as DILI and subsequently were found positive for HEV RNA by PCR.

In the industrialized world, HEV genotypes 3 and 4 are responsible for sporadic cases of autochthonous HEV infection, where the disease is zoonotic. Imported,

travel related, cases are usually genotypes 1 and 2. In this study, genotyping of 6 travel-related cases with available serum revealed genotype 1 in all cases, a genotype which is prevalent in India where all cases were acquired. One case of hepatitis E in a foreign worker from Nepal who spent one month in Israel, revealed genotype 1 as well.

Israel is located amid countries endemic for HEV, with evidence for genotype 1 in most of the cases reported from those countries. For example, isolation of HEV circulating in Egypt, a country with HEV seroprevalence among the highest in the world, revealed HEV genotype 1 in acute HEV cases, both from rural and urban areas[44-46]. Studies of North African countries revealed autochthonous infection with genotype 1 as well[2]. Data on the HEV-genotype circulating in Lebanon, Syria, and Jordan is lacking.

Unfortunately, we were unable to retrospectively test for the genotype in the autochthonous group of patients. The cause included the retrospective design of this study which did not enable storage of large volumes of suspected sera and the physical state of some of the stored sera which were thawed and frozen several times prior to an attempt of sequencing.

However, a recent Israeli study confirmed the presence of HEV in 8.2% of the 169 sewage samples tested throughout the country during 2013-2015[47]. Sequencing revealed genotype 3 in all sewage samples positive for HEV. These data along with the evidence for acute autochthonous HEV in Israel shown in the present study, suggest autochthonous infection with HEV genotype 3, as seen in Europe and in contrast to the circulating HEV genotype in Israel bordering countries.

The present study has several limitations. The information of exposure related to different risk factors, was based on patients' recollections of events that happened years before the survey, and consequently might be biased. And as mentioned, we were unable to retrospectively test for the HEV genotypes in the autochthonous group of patients. Thus phylogenetic analysis of HEV confirmed autochthonous cases in Israel remains an undertaking for the future.

This report, along with previous reports from Western countries mentioned above, provides evidence for the presence of sporadic autochthonous or travel-related acute HEV cases in the industrialized world. At present, treatment of active HEV infection with viremia with an effective anti-viral agent remains an elusive goal[48].

To date, two types of recombinant HEV vaccine have been developed[49-51], but neither of them is commercially available in the Western countries. Additionally both vaccines are genotype 1-based, and although they would be very useful in pregnant women and travelers to endemic regions, their efficacy in preventing HEV infection in non-endemic areas (where other genotypes predominate) needs to be investigated.

COMMENTS

Background

Hepatitis E virus (HEV), one of the leading causes of viral hepatitis worldwide, is transmitted predominantly *via* the fecal-oral route and responsible for epidemics of acute hepatitis in developing countries. However studies have shown that HEV is an emerging infection in industrialized countries as well, with increasing evidence of autochthonous cases (contracted locally) in addition to imported infection among immigrants and travelers from endemic countries. Israel is an industrialized country located amid endemic countries and absorbs immigrants from African countries, however a significant portion of Israel's population eats only kosher food (*i.e.*, do not eat pork, game meat or seafood), therefore the existence of autochthonous cases is not obvious.

Research frontiers

This study is a nation-wide epidemiological study of acute HEV cases diagnosed in Israel between the years 1993-2013. The aim of this manuscript was to identify whether there is any evidence of acute autochthonous HEV in Israel and to characterize the epidemiology, risk factors and clinical presentation of all documented acute HEV infection in Israel.

Innovations and breakthroughs

This report demonstrates, for the first time, the significant presence of autochthonous acute HEV in Israel (41% of all cases were autochthonous). Additionally, the imported HEV cases were from travelers and foreign workers acquiring the disease mainly in the Indian subcontinent.

Applications

The presence of autochthonous cases in Israel highlights the need for medical practitioners to be acquainted with the disease, even in non-endemic countries, and accurate diagnostic means should be easily accessible.

Terminology

"Autochthonous infection" is an infection contracted in the area where reported. Autochthonous HEV infection in Israel relates to patients with acute hepatitis E, with no history of travel during the last six months before disease onset, and who are not immigrants. In contrast, imported infection is one contracted in a country endemic for HEV, and occurs among travelers and immigrants.

Peer-review

The manuscript represents an interesting study of the etiology of acute hepatitis HEV in Israel reporting well documented data of interest to the scientific community.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Comprehensive mutation screening for 10 genes in Chinese patients suffering very early onset inflammatory bowel disease

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Author contributions: Xiao Y and Wang XQ contributed equally to this work; Xiao Y and Xu CD designed this study; Xiao Y and Wang XQ performed the next generation sequencing; Yu Y, Guo Y, Xu X and Gong L collected the patients and recorded the data; Zhou T and Li XQ analyzed the data.

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

Conflict-of-interest statement: All the authors listed declare no conflicts of interest.

Data sharing statement: Technical appendix, statistical code, and dataset is available from the corresponding author at chundixu55@163.com.

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Abstract

AIM: To perform sequencing analysis in patients with very early-onset inflammatory bowel disease (VEO-IBD) to determine the genetic basis for VEO-IBD in Chinese pediatric patients.

METHODS: A total of 13 Chinese pediatric patients with VEO-IBD were diagnosed from May 2012 and August 2014. The relevant clinical characteristics of these patients were analyzed. Then DNA in the peripheral blood from patients was extracted. Next generation sequencing (NGS) based on an Illumina-Miseq platform was used to analyze the exons in the coding regions of 10 candidate genes: *IL-10*, *IL-10RA*, *IL-10RB*, *NOD2*, *FUT2*, *IL23R*, *GPR35*, *GPR65*, *TNFSF15*, and *ADAM30*. The Sanger sequencing was used to verify the variations detected in NGS.

RESULTS: Out of the 13 pediatric patients, ten were diagnosed with Crohn's disease, and three diagnosed with ulcerative colitis. Mutations in *IL-10RA* and *IL-10RB* were detected in five patients. There were four patients who had single nucleotide polymorphisms associated with IBD. Two patients had *IL-10RA* and

FUT2 polymorphisms, and two patients had *IL-10RB* and *FUT2* polymorphisms. Gene variations were not found in the rest four patients. Children with mutations had lower percentile body weight (1.0% *vs* 27.5%, P = 0.002) and hemoglobin (87.4 g/L *vs* 108.5 g/L, P = 0.040) when compared with children without mutations. Although the age of onset was earlier, height was shorter, and the response to treatment was poorer in the mutation group, there was no significant difference in these factors between groups.

CONCLUSION: *IL-10RA* and *IL-10RB* mutations are common in Chinese children with VEO-IBD. Patients with mutations have an earlier disease onset, lower body weight and hemoglobin, and poorer prognosis.

Key words: Pediatric inflammatory bowel disease; Very early-onset inflammatory bowel disease; Interleukin 10 receptor; *NOD2* gene; *FUT2* gene

Core tip: In this small-sample size study, we performed next generation sequencing for 10 candidate genes in Chinese pediatric patients with very early onset inflammatory bowel disease. We found that *IL-10RA* and *IL-10RB* mutations were common. There were five patients harbouring mutations in these two genes and accounted for 38.5% of all samples. Besides, there were four patients who had single nucleotide polymorphisms associated with inflammatory bowel disease. Pediatric patients with mutations had an earlier disease onset, lower body weight, markedly lower hemoglobin, and poorer prognosis.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic and recurrent gastrointestinal inflammatory disease in children. Based on clinical characteristics, laboratory tests, and endoscopic and pathological presentations, IBD can be subdivided into Crohn's disease (CD), ulcerative colitis (UC), and IBD-unclassified (IBD-U)[1]. Our previous study showed that the annual incidence of IBD in the 0- to 14-year age group of Shanghai residents steadily increased from 2000 to 2010[2]. Although pediatric IBD mainly occurs in adolescence[2], approximately 15% of IBD pediatric patients have very early-onset IBD (VEO-IBD) that begins before 6 years of age, and 1% of children develop this disease before reaching 1 year of age[3,4]. The majority of VEO-IBD cases have clinical characteristics that are distinct from those of classic IBD with adult and adolescent onset. VEO-IBD has more severe clinical symptoms, resistance to a variety of immunosuppressive therapies, and a poor prognosis after conventional treatments. Some scholars even consider VEO-IBD to be a completely different disease from classic IBD[5].

Previous studies suggested that persistent intestinal immune dysfunction in a genetically susceptible individual exposed to adverse environmental factors is an important mechanism for IBD development. Genome-wide association studies (GWAS) have discovered a total of 163 loci associated with the risk for IBD development[6]. However, disease onset at an early stage of life suggests a leading role for rare gene variations in VEO-IBD patients, especially in children with a disease onset before the age of 1 year. These low frequency mutations are difficult to detect using GWAS. Next generation sequencing technology allows for the high-throughput sequencing of exons in a series of genes concurrently; therefore, rare gene variations can be discovered[7]. Since Glocker *et al*[8] first discovered in 2009 that mutations in genes encoding the α subunit (IL-10R1, encoding gene *IL-10RA*) and the β subunit (IL-10R2, encoding gene *IL-10RB*) of the interleukin-10 (IL-10) receptor could induce VEO-IBD development, a few studies have continuously discovered mutations in genes encoding IL-10R1, IL-10R2, and IL-10[5,9-12]. However, current reports are limited, and the majority of studies are small-size case studies. Reports on the Han Chinese population are scarcer[13,14].

This study used the Illumina-Miseq platform to sequence candidate genes in Han Chinese children diagnosed with VEO-IBD. The candidate genes included genes involved in the IL-10 signaling pathway, such as *IL-10*, *IL-10RA*, and *IL-10RB*, and genes highly associated with the development of CD in previous studies, including *NOD2, FUT2, IL-23R, GPR35, GPR65, TNFSF15,* and *ADAM30*. This study furthers our understanding of the genetic factors associated with VEO-IBD development in Han Chinese children.

MATERIALS AND METHODS

Patient consent and ethic committee approval

Verbal and written consent was obtained from the parents of all of children included this study. Ethic committee approval for the study was granted by Institutional Review Boards of Ruijin Hospital affiliated to Shanghai Jiao Tong University School of Medicine.

Study subjects

A total of 13 pediatric patients with repeated diarrhea, mucus and bloody stool, or abdominal pain who were diagnosed by laboratory tests and digestive endoscopy with VEO-IBD in the Pediatric Department of Ruijin

Hospital of Shanghai Jiao Tong University School of Medicine between May 2012 and August 2014 were included in this study. All of the patients were Han Chinese. VEO-IBD was defined as IBD onset before the age of 6 years, and a disease onset before 2 years of age was called infantile-onset IBD[15,16]. The clinical characteristics of these pediatric patients, including gender, age of disease onset, body height, body weight, family history, clinical symptoms, complications, major laboratory examinations, endoscopic presentations, and therapeutic effects, were retrospectively analyzed.

Laboratory and digestive endoscopic examinations

Relevant laboratory examinations, including complete blood count (CBC), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), tumor necrosis factor α (TNF-α) level, immunoglobulins G, A, M, and E, vitamin D, human Immunodeficiency virus (HIV) and human cytomegalovirus (CMV) antibody detection in serum, T lymphocyte flow cytometry sorting, stool parasite tests, stool culture, and stool *Clostridium difficile* toxin detection, were performed when the patients were admitted to the hospital. Common infectious diseases and primary immunodeficiency diseases were excluded.

All patients received a colonoscopy under general anesthesia. A biopsy of the colonic mucosa under endoscopy was performed for pathological examination.

Illumina-Miseq platform sequencing

Genomic DNA extraction: After obtaining verbal and written informed consent from the patients' parents, genomic DNA in the peripheral blood from 13 pediatric patients was extracted using a FlexiGene DNA Kit (Qiagen Inc., Germany). Another 100 copies of DNA extracted from patients suffering from idiopathic short stature (ISS) in previous research were used to test frequency of mutant sites which were newly detected in our study.

Multiplex PCR primer design: Based on the stability of the Illumina-Miseq experiment and the operability of subsequent steps, the length requirement of target fragments for sequencing was < 400 bp. If the length of an exon was longer than 400 bp, an additional pair of primers was designed with overlapping bases of adjacent fragments. To avoid a high number of non-target fragment products, primers were grouped and suspended in a primer mix before the multiplex PCR was performed. The concentration of each primer in the primer mix was 10 mmol/L. The basic requirement for grouping was the lack of matching sequences between two of the amplified products. Oligo 7 software was used to design primers for exons of the encoding region of the 10 candidate genes: *IL-10, IL-10RA, IL-10RB, NOD2, FUT2, IL23R, GPR35, GPR65, TNFSF15,* and *ADAM30*. A total of 86 pairs of primers were designed. The sequences are shown in supplementary Table 1.

Multiplex PCR amplification of candidate genes: A Qiagen Multiple PCR Kit was used in this study. The PCR amplification reaction system had a total volume of 21 μL, including 4 μL of ddH_2O, 2 μL of Q-solution (5 ×), 4 μL of 10 mmol/L primer mix, 10 μL of buffer mix, and 1 μL of the DNA template (20 ng/μL). The reaction procedure consisted of pre-denaturation at 94 ℃ for 15 min, denaturation at 94 ℃ for 40 s, annealing at 63 ℃ for 1 min, and extension at 72 ℃ for 40 s. After each cycle, the annealing temperature was reduced by 0.5 ℃ for 10 cycles until the annealing temperature reached 58 ℃. Next, the amplification was continued for 30 cycles with a constant annealing temperature of 58 ℃. The final extension at 72 ℃ lasted for 10 min. The PCR products were stained with 100 × GelRed and subjected to 1% agarose electrophoresis (120 V for 60 min).

The purified multiplex PCR products were sent to Shanghai South Gene Technology Co., Ltd. for sequencing analysis with the Illumina-Miseq platform. After sequencing, the nucleotide sequence information was compared with the standard gene sequences available in GenBank. The obtained gene mutation sites were compared with information in the dbSNP, HGMD, and OMIM databases to determine if the mutations had been previously reported.

To confirm the accuracy of the results, the corresponding gene sequences for the mutations discovered using the Illumina-Miseq platform were sequenced again using the Sanger sequencing method.

The newly discovered gene variation sites were analyzed to predict their influence on protein functions using two online databases: SIFT (http:// http://sift.jcvi.org/) and PolyPhen 2 (http://genetics.bwh.harvard.edu/pph2/).

Statistical analysis

According to the sequencing results, the 13 pediatric patients were divided into two groups. The patients who harboured pathogenic mutations were in group 1. Those without pathogenic mutations (including presence of polymorphisms only or wild type) were in group 2. The differences in diagnosis, age of disease onset, growth indicators (percentiles of body weight and height were calculated according to WHO standards), complications (perianal diseases and recurrent infection), and therapeutic effects among all groups were compared. Because the sample size was small, quantitative and ranked ordinal data were subjected to nonparametric statistics. The Mann-Whitney Test was performed, and the difference was statistically analyzed using exact probability. SPSS13.0 for Windows software was used for the statistical analysis. $P < 0.05$ indicated a significant difference.

Table 1 Genotypes of 13 patients diagnosed with very early-onset inflammatory bowel disease

Patient	Gene	Variation	Homo/Heterozygote	Function defect
1	*IL-10RA*	p.R101W	Homozygote	Yes
2	*IL-10RA*	p.R101W	Compound	Yes
		p.V100G (novel mutation)	heterozygote	Pathogenic supporting by Polyphen 2 and SIFT
3	*IL-10RA*	p.R101W	Compound	Yes
		p.Y64C (novel mutation)	heterozygote	Pathogenic supporting by Polyphen 2 and SIFT
4	*IL-10RA*	p.R117H (rs199989396)	Heterozygote	Yes
	NOD2	p.R703C (rs5743277)	Heterozygote	Susceptibility to CD recorded in HGMD
	FUT2	p.I140F (rs1047781)	Heterozygote	Susceptibility to CD in Chinese population reported by Hu *et al*[31]
5	*IL-10RB*	p.K47E (rs2834167)	Homozygote	SNP in a VEO-UC child reported by Galatola *et al*[29]
		p.E141K (rs387907326)	Heterozygote	Pathogenic supporting by Polyphen 2 and SIFT
	IL-10RA	p.P115P (rs22280554)	Homozygote	Susceptibility to VEO-IBD reported by Moran *et al*[30]
		p.I224V (rs22280555)	Homozygote	
	FUT2	p.I140F (rs1047781)	Heterozygote	Susceptibility to CD in Chinese population reported by Hu *et al*[31]
6	*IL-10RA*	p.P115P (rs22280554)	Homozygote	Susceptibility to VEO-IBD reported by Moran *et al*[30]
		p.I224V (rs22280555)	Homozygote	
	FUT2	p.I140F (rs1047781)	Homozygote	Susceptibility to CD in Chinese population reported by Hu *et al*[31]
7	*IL-10RA*	p.P115P (rs22280554)	Homozygote	Susceptibility to VEO-IBD reported by Moran *et al*[30]
		p.I224V (rs22280555)	Homozygote	
	FUT2	p.I140F (rs1047781)	Heterozygote	Susceptibility to CD in Chinese population reported by Hu *et al*[31]
8	*IL-10RB*	p.K47E (rs2834167)	Homozygote	SNP in a VEO-UC child reported by Galatola *et al*[29]
	FUT2	p.I140F (rs1047781)	Heterozygote	Susceptibility to CD in Chinese population reported by Hu *et al*[31]
9	*IL-10RB*	p.K47E (rs2834167)	Heterozygote	SNP in a VEO-UC child reported by Galatola *et al*[29]
	FUT2	p.I140F (rs1047781)	Heterozygote	Susceptibility to CD in Chinese population reported by Hu *et al*[31]

Patients 10, 11, 12 and 13 were wild types in all these genes. CD: Crohn's disease; UC: Ulcerative colitis; VEO-IBD: Very early-onset inflammatory bowel disease; VEO-UC: Very early-onset ulcerative colitis; SNP: Single nucleotide polymorphism; HGMD: The Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php).

RESULTS

Genotyping in VEO-IBD patients

_IL-10RA_, _IL-10RB_, and _IL-10_ mutations: *IL-10RA* mutations were detected in four patients, an *IL-10RB* mutation was detected in one patient, and an *IL-10* mutation was not detected in any of the 13 patients.

The detected *IL-10RA* mutations were all in exon 3: c.A191G (p.Y64C), c.T299G (p.V100G), c.C301T (p.R101W), and c.G350A (rs199989396) (p.R117H). The p.R101W mutation was the most common and was detected in three patients (patients 1-3). The other mutations were detected in only one patient. Patient 1 had a homozygous mutation, patients 2 and 3 had compound mutations, and patient 4 had a heterozygous mutation (Table 1 and Figure 1).

Among detected *IL-10RA* mutations, p.Y64C and p.V100G were new mutations that were predicted to be deleterious by SIFT and Polyphen 2. These novel mutant sites were not found in 100 ISS children. The other two mutations had been confirmed to be deleterious in several studies[5,12,14,17].

An *IL-10RB* heterozygous mutation was detected in one patient (patient 5) (Table 1 and Figure 1). This c.G421A (p.E141K) (rs387907326) mutation was located in exon 4 and was also predicted as a deleterious mutation by SIFT and Polyphen 2. A nonsense mutation in the same site was detected in previous studies[11,18].

Candidate gene polymorphisms

After the sequence analysis of the coding regions of 10 candidate genes, we found that six patients (patient 4, 5, 6, 7, 8 and 9) had many IBD-associated single nucleotide polymorphisms (SNPs) in *IL-10RA*, *IL-10RB*, *NOD2*, and *FUT2*. The SNP loci in *IL-10RA* were rs22280554: c.G525A, p. P175P and rs22280555: c.A670G, p.I224V; the SNP locus in *IL-10RB* was rs2834167: c.A139G, p.K47E; the SNP locus in *NOD2* was rs5743277: c.C2107T, p.R703C; and the SNP locus in *FUT2* was rs1047781: c.A418T, p.I140F (Table 1).

In addition to the detected p.R117H heterozygous mutation in *IL-10RA*, patient 4 also had heterozygous SNPs in *NOD2* and *FUT2*.

Patient 5 had a heterozygous p.E141K mutation (rs387907326) in *IL-10RB* and SNPs in *IL-10RA, IL-10RB,* and *FUT2*. The SNP loci in *IL-10RA* were rs22280554 and rs22280555. The homozygous SNP loci for *IL-10RB* were rs2834167. The SNP in *FUT2* was heterozygous.

Patients 6 and 7 had SNPs in *IL-10RA* and *FUT2*. Patients 8 and 9 had SNPs in *IL-10RB* and *FUT2*.

Four patients did not show any IBD-associated variations in the coding regions of the 10 candidate genes.

There was no IBD-associated variation discovered in the coding regions of six genes: *IL-10, IL-23R*, *GPR35*, *GPR65*, *TNFSF15*, and *ADAM30*.

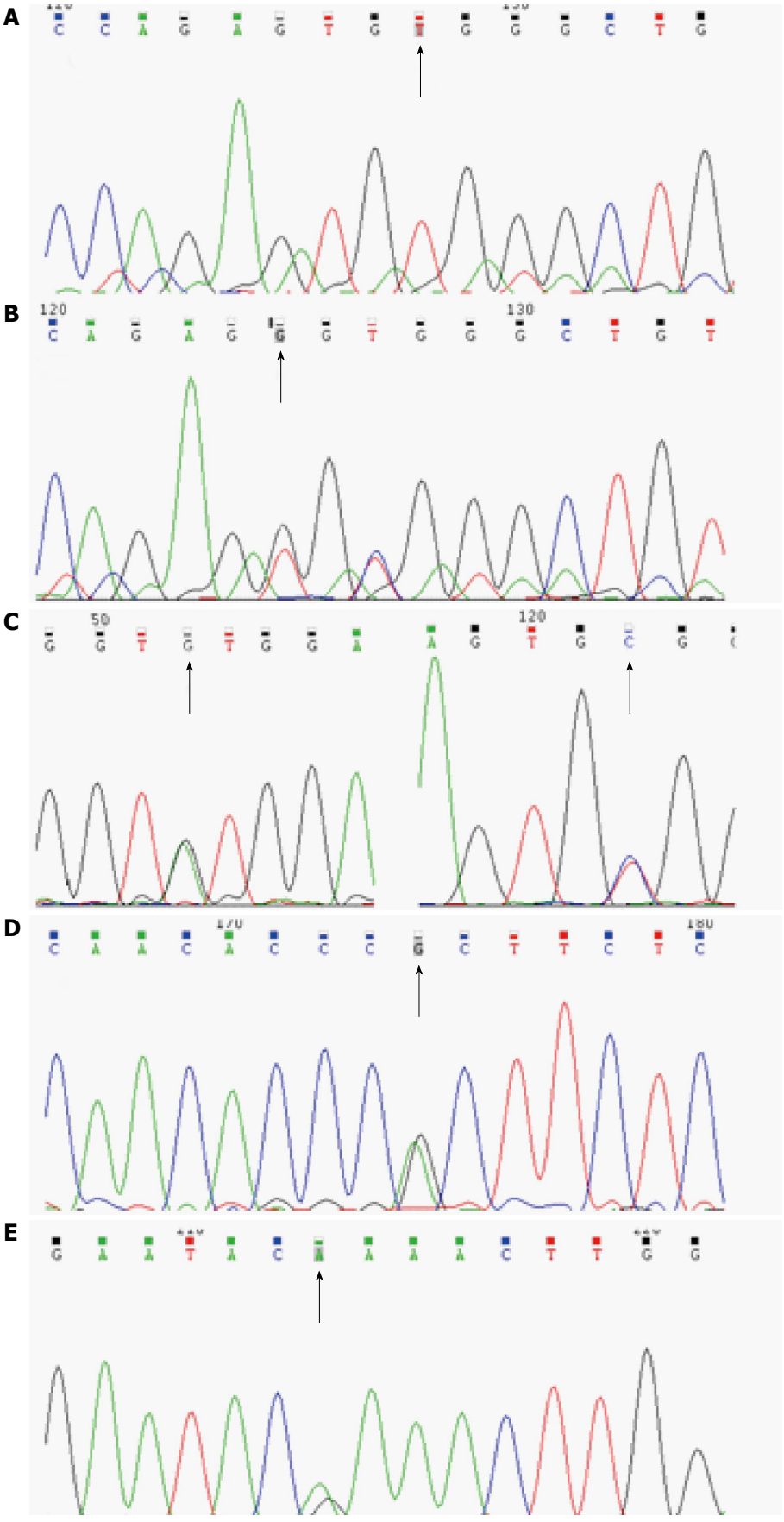

Figure 1 Causative mutations in *IL-10RA* (A-D) or *IL-10RB* (E). A: Patient 1, c.C301T, p.R101W, homozygote; B: Patient 2, c.T299G, p.V100G and c. C301T, p.R101W, compound heterozygote; C: Patient 3, c.A191G, p.Y64C and c. C301T, p.R101W, compound heterozygote; D: Patient 4, c.G35A, p.R117H (rs199989396), heterozygote; E: Patient 5, c.G421A, p.E141K (rs387907326), heterozygote.

Clinical characteristics of VEO-IBD pediatric patients

Out of the 13 VEO-IBD pediatric patients in this study, ten were diagnosed with CD (M:F = 9:1) and three had UC (M:F = 1:2). The mean age of disease onset was 5.8 ± 9.7 mo (range: birth to 3 years of age). None of the parents of the patients had a consanguineous marriage. Patient 8 had a brother that died as a neonate because of repeated diarrhea after birth. There was no clear diagnosis made at that time. The clinical symptoms of the pediatric patients included repeated abdominal pain (13/13), diarrhea (11/13), mucus and bloody stool (11/13), failure to thrive (8/13), recurrent infection (7/13), and perianal fistulas and abscesses (5/13). The colonoscopic presentation of patients with causative mutations showed pancolitis, cobblestone-like changes in mucosa, and deep and large ulcers (Figure 2). All patients received immunosuppressive treatment with glucocorticoids, 6-mercaptopurine and/or infliximab, and thalidomide; however, varying therapeutic effects were observed. Two patient died from severe sepsis or intestinal failure, 2 patients showed no change, 4 patients showed a partial alleviation of symptoms, and 5 patients showed complete clinical remission (Table 2).

Clinical characteristics of different genotypes

Based on the presence of causative mutations in *IL-10RA* and *IL-10RB*, 13 patients were divided into two groups for analysis (group 1: causative mutations in *IL-10RA* or *IL-10RB*; group 2: polymorphisms and no causative mutations). The five patients in group 1 were all diagnosed with CD (100%). Four of these patients had recurrent infections (80%), and three patients had perianal diseases (60%). In group 2 (eight patients), five patients were diagnosed with CD (62.5%), and the other three patients were diagnosed with UC (37.5%). There were only three (37.5%) and two (25%) patients that had recurrent infections and perianal diseases, respectively. Patients in group 1 had lower body weight percentile (1.0% *vs* 27.5%, $P = 0.002$) and hemoglobin concentrations (87.4 g/L *vs* 108.5 g/L, $P = 0.040$) when compared with group 2. Although patients in group 1 had a younger age of disease onset (2.7 mo), lower body height percentile (5.0%), and higher CRP (60.7 mg/L), there were no significant differences when compared with group 2 (Table 3).

DISCUSSION

The currently recognized pathogenetic mechanism of IBD is the involvement of many environmental triggers and genetic susceptibility that causes intestinal immune dysfunction. However, the influence of genes are likely more important than environmental factors for VEO-IBD patients with a disease onset prior to 6 years of age, especially for patients with an infantile onset prior to 1 year of age[19]. GWAS studies suggested that SNPs of *IL-10* and *STAT3* were associated with IBD[20-23]. Previous studies confirmed that IL-10 or IL-10 receptor gene knockout mice had severe chronic inflammation of the intestinal tract[24]. IL-10 forms a complex with two molecules of IL-10R1 and two molecules of IL-10R2 to activate Janus kinase 1 (Jak1) and tyrosine kinase 2 (Tyk2). This activation results in the phosphorylation of signal transducer and activator of transcription 3 (STAT3), which regulates the transcription of specific genes. Studies suggested that IL-10-mediated signals effectively reduced the number of Th17 cells and relieved intestinal inflammation in CD[25]. These data indicated that the anti-inflammatory IL-10 signaling pathway plays a critical role in the regulation of intestinal immune homeostasis.

Since Glocker *et al*[8] first reported in 2009 that gene mutations in *IL-10RA* and *IL-10RB* caused infantile onset IBD[8], studies have continuously reported mutations in *IL-10*, *IL-10RA*, and *IL-10RB* in patients with infantile onset IBD[9-12,18,26]. In these limited data, the majority of patients were Arabian or Caucasian and the offspring of a consanguineous marriage. There are few reports on the Han Chinese population, which included only three pediatric patients to date[13,14].

In this study, we used high-throughput next generation sequencing technology to sequence 10 IBD-associated genes, *IL-10*, *IL-10RA*, *IL-10RB*, *NOD2*, *FUT2*, *IL-23R*, *GPR35*, *GPR65*, *TNFSF15*, and *ADAM30*, in 13 Han Chinese children diagnosed with VEO-IBD. A total of four mutations were discovered in *IL-10RA*, including two novel mutations. There was one mutation in *IL-10RB*. These pathogenic mutations were found in five patients, which accounted for 38.5% of all VEO-IBD cases. Among these patients, one had an *IL-10RA* homozygous mutation, two had *IL-10RA* compound heterozygous mutations, one had an *IL-10RA* heterozygous mutation, and one had an *IL-10RB* heterozygous mutation. All *IL-10RA* mutations were in exon 3, and c.C301T (p.R101W) showed the highest frequency. The c.C301T (p.R101W) and c.G350A (p.R117H) mutations in *IL-10RA* were previously reported in similar pediatric patients. These mutations may disrupt signal transduction after activation of the IL-10 receptor; therefore, STAT3 is not phosphorylated and intractable inflammatory reactions in the intestinal tract of pediatric patients develop[5,12]. The two novel mutations in *IL-10RA* discovered in this study were c.A191G (p.Y64C) and c.T299G (p.V100G). Because of condition limitations, we did not perform functional studies on these mutations. However, the SIFT prediction results for these two mutations were deleterious (scores of 0 and 0.002, respectively), and the Polyphen 2 prediction results were probably damaging (both scores were 1.000). These predictions suggest that these two mutations are pathogenic. According to the recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology[27], these two

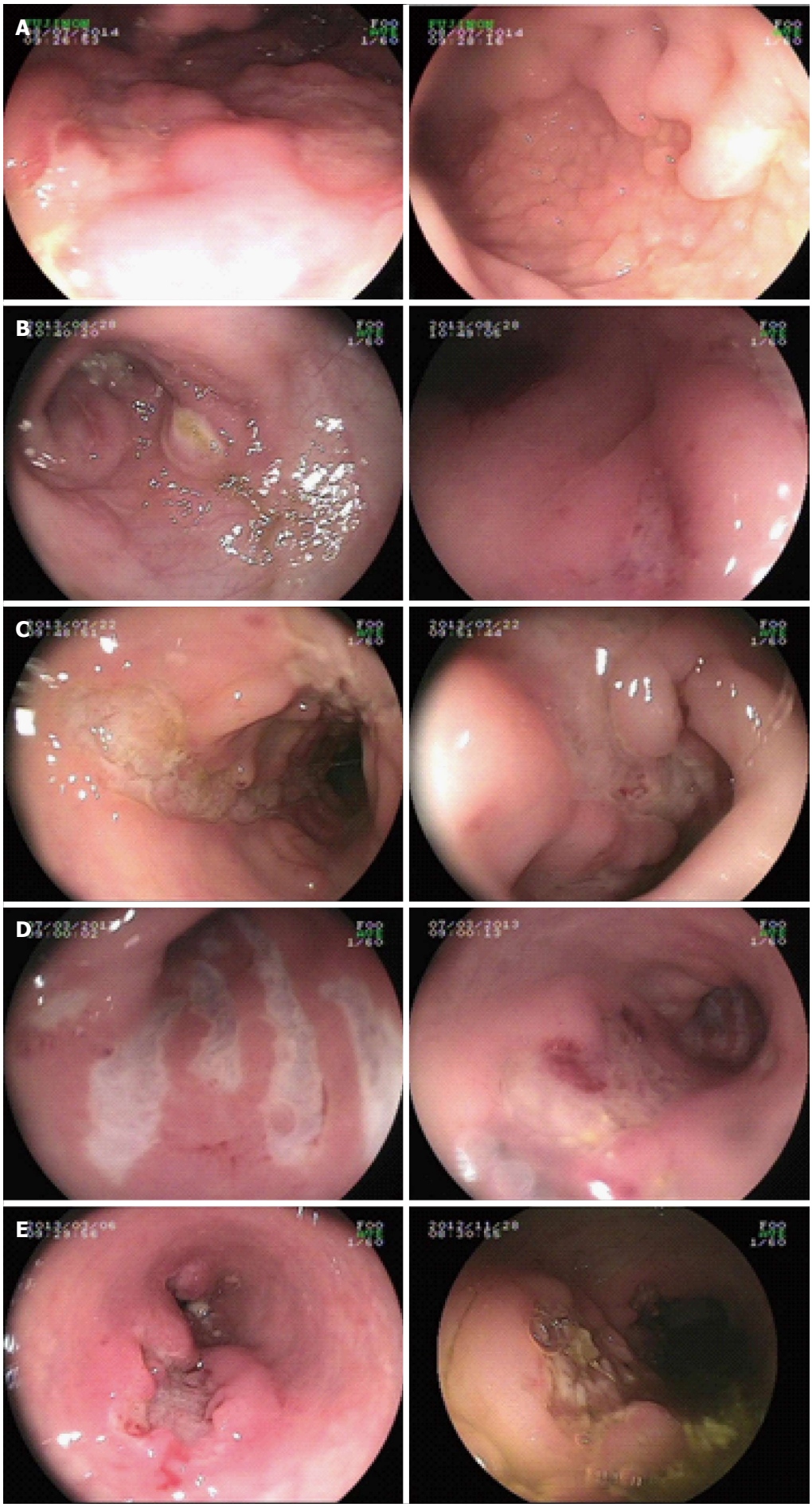

Figure 2 Colonoscopic presentation of patients with causative mutations showed pancolitis, cobblestone-like changes in mucosa, and deep and large ulcers. A to E presents patient 1 to patient 5, respectively.

Table 2 Clinical manifestations of very early-onset inflammatory bowel disease

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9	Patient 10	Patient 11	Patient 12	Patient 13
Gender	F	M	M	M	M	M	M	M	M	M	M	F	F
Age of onset (mo)	8	1	0.3	0.3	4	0.2	9	2	0.5	3	0.7	10	36
Height percentile	19%	1%	3%	1%	1%	1%	52%	1%	15%	1%	19%	16%	20%
Weight percentile	1%	1%	1%	1%	1%	20%	55%	13%	8%	15%	16%	60%	33%
Diarrhea (times/d)	> 10	7-8	> 10	10	5-10	5-6	7-8	2-4	7-8	No diarrhea	7-8	No diarrhea	2-3
Bloody stool	+	+	+	+	+	-	+	+	+	-	+	+	+
Infection	Sepsis	Pneumonia	No	Pneumonia, *Clostridium difficile* infection	Sepsis, oral candidiasis, fungemia, *Clostridium difficile* infection	Recurrent respiratory infection	No	No	No	Repeated fever of unknow origin	Oral candidiasis, gingivitis	No	No
Perianal lesion	Fistulae	No	No	Excrescence	Fistulae, abscess, excrescence	Fistulae, ulcer	No	No	No	No	Fistulae, abscess, excrescence	No	No
Clinical diagnosis	CD	CD	CD	CD	CD	CD	CD	CD	UC	CD	CD	UC	UC
Medication	GC, 6-MP	GC, IFX, THD	GC, THD	GC, IFX[1], THD	GC, IFX, THD	GC, IFX[1]	GC, IFX, MES	GC, IFX[1], THD, 6-MP	GC, MES	GC, 6-MP, THD	GC, IFX, THD, 6-MP	MES	GC, MES
Clinical status	NR	PR	Died at 2 yr because of severe sepsis	PR	Died at 3 yr because of intestinal failure	NR	CR	PR	CR	CR	PR	CR	CR

[1]Allergic to IFX. CD: Crohn's disease; UC: Ulcerative colitis; GC: Glucocorticoid; 6-MP: 6-mercaptopurine; IFX: Infliximab; THD: Thalidomide; MES: Mesalazine; NR: Non-remission; PR: Partial remission; CR: Complete remission.

mutations were defined as pathological supporting. Therefore, we speculate that these two novel mutations individually formed compound heterozygotes with the c.C301T (p.R101W) mutation to cause the disease symptoms observed in patients 2 and 3.

Previous analyses showed that the colitis caused by gene mutations in IL-10 and its receptor exhibited an autosomal recessive inheritance pattern. In the current study, patients 4 and 5 were carriers of heterozygous mutations in *IL-10RA* and *IL-10RB*, respectively. The c.G350A (p.R117H) mutation in *IL-10RA* carried by patient 4 was a pathogenic mutation[5,12,17]. The c.G421A (p.E141K) mutation in *IL-10RB* carried by patient 5 may affect protein function as predicted by SIFT (score = 0.026) and Polyphen 2 (score = 0.946). However, the clinical presentation of these two patients was similar to the symptoms of patients with other IL-10 receptor mutations: disease onset within 1 year of age, the presence of perianal diseases and recurrent infection, and resistance to conventional medication treatment. Based on currently available knowledge, there are at least 50 single-gene genetic conditions that induce IBD-like diseases, and the majority of conditions are related to immunodeficiency[4,6]. Therefore, the two patients that did not conform to a Mendelian genetic pattern might also carry abnormal sites on other genes that cause the disease symptoms. In addition to carrying a pathogenic mutation in *IL-10RA*, patient 4 also had a non-synonymous SNP (nsSNP): rs5743277 in *NOD2*. SIFT prediction results suggest that the nsSNP is deleterious (score = 0), and the Polyphen 2 prediction results suggest the nsSNP is probably damaging (score = 0.999). This polymorphism was already present in the HGMD database and has been considered to cause susceptibility to CD[28]. Patient 5 had a similar condition. In addition to carrying an *IL-10RB* mutation, patient 5 also had multiple polymorphisms: rs22280554 (homozygous) and rs22280555 (homozygous) in *IL-10RA*,

Table 3 Comparison of features between patients with mutations and polymorphisms

	Group 1	Group 2
Size of sample	5	8
Age of onset (mo)	2.7	7.7
Height percentile	5.0%	15.6%
Weight percentile[a]	1.0%	27.5%
WBC ($\times 10^{9}$)	15.2	16.3
Hemoglobin (g/L)[a]	87.4	108.5
Platelets ($\times 10^{9}$)	538.4	424.0
C reactive protein (mg/L)	60.7	35.9
ESR (mm/H)	32.2	16.6
TNFα (pg/mL)	44.5	51.6
Diagnosis of CD	100.0%	62.5%
Recurrent infection	80.0%	25.0%
Perianal disease	60.0%	25.0%

[a]$P < 0.05$. All measurement data are expressed as mean. Group 1: Mutations in *IL-10RA* or *IL-10RB*; Group 2: Polymorphisms. Height and weight percentile was calculated according to WHO charts. WBC: White blood cell; ESR: Erythrocyte sedimentation rate; TNFα: Tumor necrosis factor alpha; CD: Crohn's disease.

rs2834167 (homozygous) in *IL-10RB*, and rs1047781 (heterozygous) in *FUT2*. There are previous reports on the pathogenicity of these SNPs. For example, Galatola *et al*[29] reported that the heterozygous rs2834167 in *IL-10RB* and the heterozygous mutation in the promoter region of *IL-10RA* caused the development of UC in an 18-month-old patient. Although rs22280554 did not cause a change in the amino acid sequence of IL-10R1, a study by Moran *et al*[30] showed that rs22280554 and rs2228055 in *IL-10RA* may increase the risk for VEO-IBD, especially VEO-UC. Furthermore, in the Han Chinese population, the rs1047781 polymorphism in *FUT2* may increase the risk for CD development[31]. The above SNPs were also detected in four patients in this study. Therefore, their disease development may be due to "trans-heterzygous": the collective effects of a variety of detected mutations. Another possible cause is that the pathogenic genes were not detected in this study.

When genotypes and phenotypes were combined for analyses, the results showed that the disease phenotype in patients with mutations were more severe. The age of disease onset was earlier, the patients were more likely to have combined recurrent infections and perianal diseases, their body weight and height were low, anemia was more severe, inflammatory indicators were high, and the prognosis was much poorer. These results are in accordance with previous studies[5,8-14,18,32]. However, the sample size of this study was small, and significant differences were only found in body weight and hemoglobin parameters. Because of the influence of cultural ideas, family members find difficulty in accepting an ileostomy as a disease treatment. Past literature reported that pediatric patients with *IL-10RA* and *IL-10RB* mutations could be cured through hematopoietic stem cell transplantation[4,6,8,17]; therefore, some patients are waiting for a donor match.

In this study, we found that mutations in *IL-10RA* and *IL-10RB* were more common in Han Chinese VEO-IBD patients and accounted for 38.5% of all VEO-IBD cases. The high percentage is probably due to the small number of patients in the cohort as most of our patients who were referred by other clinical IBD centers were very ill. There was a selection bias. Because VEO-IBD is relatively rare, multi-center studies on the relationship between genotypes and phenotypes in VEO-IBD patients in China are necessary. The implementation of hematopoietic stem cell transplantation therapy is the focus in research agenda.

COMMENTS

Background

Very early-onset inflammatory bowel disease (VEO-IBD) may have stronger genetic contribution. Recently, a few studies on genetic defects in the IL-10 signaling pathway have provided new insights into IBD, especially in VEO-IBD. Furthermore, a lot of genes associated with IBD were identified, such as *NOD2*, *FUT2*, *IL-23R*, *GPR35*, *GPR65*, *TNFSF15*, and *ADAM30*. Because of different genetic background, this study was set to disclose whether mutations in these genes contributed to VEO-IBD in Chinese children.

Research frontiers

In addition to the polygenic variants associated with IBD, there are rare monogenic disorders, including many immunodeficiencies that can present with IBD-like intestinal inflammation, especially in early life.

Innovations and breakthroughs

To our knowledge, this is the first cohort study to apply NGS in 13 Chinese pediatric patients with VEO-IBD to discover gene variations in these children. The result revealed that IL-10RA and IL-10RB mutations were common in Chinese VEO-IBD, especially in infantile IBD. These monogenic IBD patients had more severe clinical features.

Applications

According to the results of this study and previous studies of VEO-IBD, the authors suggest that screening for gene mutations in IL-10 signaling pathway is necessary.

Peer-review

The clinical study is focused on gene mutation analysis in VEO-IBD by NGS. The authors conclude that mutations in the IL-10 pathway are common in VEO-IBD.

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ORIGINAL ARTICLE

Retrospective Cohort Study

miR-422a is an independent prognostic factor and functions as a potential tumor suppressor in colorectal cancer

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Author contributions: Zheng GX and Wang CX conceived the study, participated in its design and coordination and helped draft the manuscript; Zheng GX and Qu AL performed the experiments and analyses; Yang YM, Zhang X and Zhang SC were responsible for collection of samples and acquiring of clinical data; all authors read and approved the final manuscript.

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Abstract

AIM: To determine the expression of miR-422a in colorectal cancer (CRC) tissues and to further explore the prognostic value and function of miR-422a in CRC carcinogenesis.

METHODS: miR-422a expression was analyzed in 102 CRC tissues and paired normal mucosa adjacent to carcinoma by quantitative real-time PCR. The relationship of miR-422a expression with clinicopathological parameters was also analyzed. Kaplan-Meier analysis and Cox multivariate analysis were performed to estimate the potential role of miR-422a. Cell proliferation, migration, and invasion were used for *in vitro* functional analysis of miR-422a.

RESULTS: The levels of miR-422a were dramatically reduced in CRC tissues compared with normal mucosa ($P < 0.05$), and significantly correlated with local invasion ($P = 0.004$) and lymph node metastasis ($P < 0.001$). Kaplan-Meier survival and Cox regression multivariate analyses revealed that miR-422a expression (HR = 0.568, $P = 0.015$) and clinical TNM stage (HR = 2.942, $P = 0.003$) were independent prognostic factors

for overall survival in CRC patients. Furthermore, *in vitro* experiments showed that overexpression of miR-422a inhibited the proliferation, migration, and invasion of SW480 and HT-29 cells.

CONCLUSION: Down-regulation of miR-422a may serve as an independent prognosis factor in CRC. MiR-422a functions as a tumor suppressor and regulates progression of CRC.

Key words: Colorectal cancer; MicroRNA; miR-422a; Prognosis; *In vitro* function

Core tip: In the present study, we found that miR-422a was dramatically reduced in colorectal cancer (CRC) tissues, and significantly correlated with local invasion and lymph node metastasis. miR-422a expression and clinical TNM stage were independent prognostic factors for overall survival in CRC patients. Furthermore, *in vitro* experiments showed that overexpression of miR-422a inhibited the proliferation, migration, and invasion of SW480 and HT-29 cells. These results indicated that down-regulation of miR-422a might serve as an independent prognosis factor in CRC, and miR-422a functions as a tumor suppressor and regulates progression of CRC.

Zheng GX, Qu AL, Yang YM, Zhang X, Zhang SC, Wang CX. miR-422a is an independent prognostic factor and functions as a potential tumor suppressor in colorectal cancer. *World J Gastroenterol* 2016; 22(24): 5589-5597 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i24/5589.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i24.5589

INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignant tumors with increasing incidence and mortality over the past several decades[1]. Despite the significant progress made in diagnostic methods and improved treatment strategies, the prognosis of CRC patients remains poor, especially in those with advanced CRC[2,3]. CRC carcinogenesis is associated with multiple alterations in oncogenes and tumor suppressor genes. A growing number of studies have revealed that microRNAs (miRNAs) might regulate up to 30% of human genes and play a pivotal role in various cellular processes including proliferation, differentiation, apoptosis, migration, and invasion[4-8]. While the functional mechanisms of miRNAs remain largely unknown in the pathogenesis of CRC, dysregulated expression of miRNAs can serve as potential biomarkers for the diagnosis and prognosis of cancer[9-11].

Several studies have determined the importance of miR-422a in human diseases such as cancer, multiple sclerosis, and postmenopausal osteoporosis. Gougelet *et al*[12] reported that miR-422a could inhibit signaling pathways regulating tumor cell proliferation in osteosarcoma. A study by Mao *et al*[13] demonstrated that miR-422a targeted key mismatch repair protein (MutLα) by suppressing MLH1 expression, resulting in genome instability and tumorigenesis. Faltejskova *et al*[14] reported that dysregulation of miR-378, miR-375, miR-422a, miR-215 and miR-135b in CRC patients played an important role in CRC pathogenesis. However, further study is needed to confirm whether miR-422a is an independent predictive factor in patients with CRC. Moreover, the functional mechanism by which miR-422a regulates CRC progression and whether it can serve as a prognostic biomarker in CRC remain largely unknown.

Previously, we have identified a serum 4-miRNA panel that included miR-19a-3p, miR-92a-3p, miR-223-3p, and miR-422a. This miRNA panel served as biomarkers for early diagnosis of colorectal adenocarcinoma[15,16]. In this study, we showed that the expression of miR-422a is dysregulated in CRC tissues. The correlation between miR-422a and certain clinical characteristics, as well as its potential as a prognostic marker for CRC, was also investigated. The effects of miR-422a on CRC cell proliferation, invasion, and migration were also assessed.

MATERIALS AND METHODS

Study population and sample collection

All written informed consent was obtained from every participant for use of tissue samples. This project was approved by the Clinical Research Ethics Committee of Qilu Hospital of Shandong University. All CRC patients were recruited from the Department of General Surgery, Qilu Hospital of Shandong University between November 2004 and December 2013. The diagnosis of CRC was confirmed by histopathology or histobiopsy. A total of 102 CRC tissues were collected and the adjacent normal mucosa tissues were used as controls since CRC is a malignant epithelial tumor and originates from glandular epithelium of the colorectal mucosa. All tissues were immediately frozen in liquid nitrogen and stored at -80 ℃ until RNA extraction. The median follow-up period for patients enrolled in this study was 63 mo (range, 14-78 mo).

Cell culture

SW480 and HT29 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The two cell lines were cultured in DMEM medium supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, United States) at 37 ℃ in an incubator containing 5% CO_2.

Table 1 Relationship between miR-422a and clinical parameters in colorectal cancer tissues

Clinical parameter	Number of cases (n = 102)	miR-422a expression, median (min-max)	P value
Gender			0.704
Male	63	1.446 (0.074-130.774)	
Female	39	1.617 (0.167-33.543)	
Age (yr)			0.328
≤ median	49	1.637 (0.088-130.774)	
> median	53	1.715 (0.074-21.871)	
Tumor location			0.092
Colon	58	1.798 (0.088-130.774)	
Rectum	44	1.887 (0.074-33.543)	
Differentiation			0.150
Well	12	1.798 (0.074-130.744)	
Moderate	67	1.922 (0.166-21.871)	
Poor	23	2.088 (0.088-20.406)	
Tumor size			0.527
≤ 5 cm	64	2.127 (0.074-130.744)	
> 5 cm	38	2.353 (0.167-21.871)	
Local invasion			0.004
T1–T2	55	3.257 (0.206-130.744)	
T3–T4	47	1.617 (0.074-20.406)	
Lymph node metastasis			0.002
No	70	2.863 (0.359-130.744)	
Yes	32	1.445 (0.074-20.406)	
TNM stage			< 0.001
Ⅰ	23	3.877 (0.265-15.999)	
Ⅱ	42	3.253 (0.074-13.566)	
Ⅲ	37	1.445 (0.329-130.744)	

Values are median (IQR: Inter-quartile range). TNM: Tumor node metastasis.

Cell transfection

CRC cells were seeded at 2 × 10^5 cells/well in 6-well plates until 30%-50% confluency and then were transfected with miR-422a mimics or negative control mimics (RiboBio, Guangzhou, China) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's introduction. The transfected cells were normalized and used in subsequent assays. The transfection efficiency was determined by RT-qPCR to verify the success of transfection.

RNA preparation, cDNA synthesis and RT-qPCR

Total RNA from cell lines, CRC tissues and normal mucosa was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, United States). The cDNA was reverse-transcribed at 65 ℃ for 5 min in a 12 μL reaction system including 1 μg RNA, 1 μL reverse transcription primer (RiboBio, Guangzhou, China), 1 μL U6 reverse transcription primer and 1 μL dNTP. Then, 4 μL buffer, 2 μL DTT and 1 μL RNase inhibitor were added and incubated at 37 ℃ for 2 min followed by adding 1 μL MMLV and incubating at 37 ℃ for 50 min and 70 ℃ for 5 min. The PCR reaction was performed as follows: 95 ℃ for 1 min, and 45 cycles of 95 ℃ for 15 s, 60 ℃ for 30 s, and 72 ℃ for 45 s. U6 was used as a reference gene and each test was performed in triplicate in this study. RT-qPCR reactions were carried out on ABI Prism 7500 System (Applied Biosystems, Foster City, CA, United States).

Cell proliferation assay

miR-422a mimics and control mimics were transfected into SW480 and HT29 cells, then cultured for another 24, 48, 72, or 96 h. Cell proliferation rates was measured with CCK-8 reagent (Beyotime, Hangzhou, China) following the manufacturer's protocol. A microplate photometer (Multiskan FC, Thermo scientific, Shanghai, China) was used to determine the optical density at 450 nm.

Transwell migration and invasion assays

Transwell migration assay was performed using transwell chambers (Corning Costar, United States). After transfection for 24 h, cells were transferred into the upper chamber. RPMI 1640 medium containing 10% FBS functioning as a chemoattractant was added to matched lower chamber. SW480 and HT29 cells unable to migrate were removed from the upper surface of the transwell membrane after incubation for 48 h. Cells that were able to invade on the lower membrane surface were fixed in methanol, stained with 0.1% crystal violet, and counted using an inverted microscope (Olympus, Tokyo, Japan). For invasion assay, the inserts were pre-coated with matrix gel (BD Biosciences, Franklin Lakes, NJ, United States).

Statistical analysis

Data were analyzed using SPSS 17.0 software (IBM Corporation, Armonk, NY, United States). The expression of miR-422a between groups was compared using Mann-Whitney U test. Kaplan-Meier method was used for overall survival analysis. The Cox regression model was used for univariate and multivariate analyses to estimate the prognostic factors.

RESULTS

Expression of miR-422a in CRC tissues and correlative analysis with clinicopathological parameters

We detected the expression of miR-422a in 102 pairs of CRC tissues and normal mucosa adjacent to carcinoma using RT-qPCR. The results indicated that miR-422a levels were significantly lower in CRC tissues compared with normal mucosa (P < 0.05) (Figure 1A). Moreover, 65.7% (67 of 102) of CRC tissues had at least 2-fold down-regulated expression of miR-422a compared with normal mucosa (Figure 1B). The levels of miR-422a in stages Ⅰ and Ⅱ samples were significantly higher than those in stage Ⅲ samples (P < 0.05). There was no significant difference in miR-422a expression between stages Ⅰ and Ⅱ samples (P

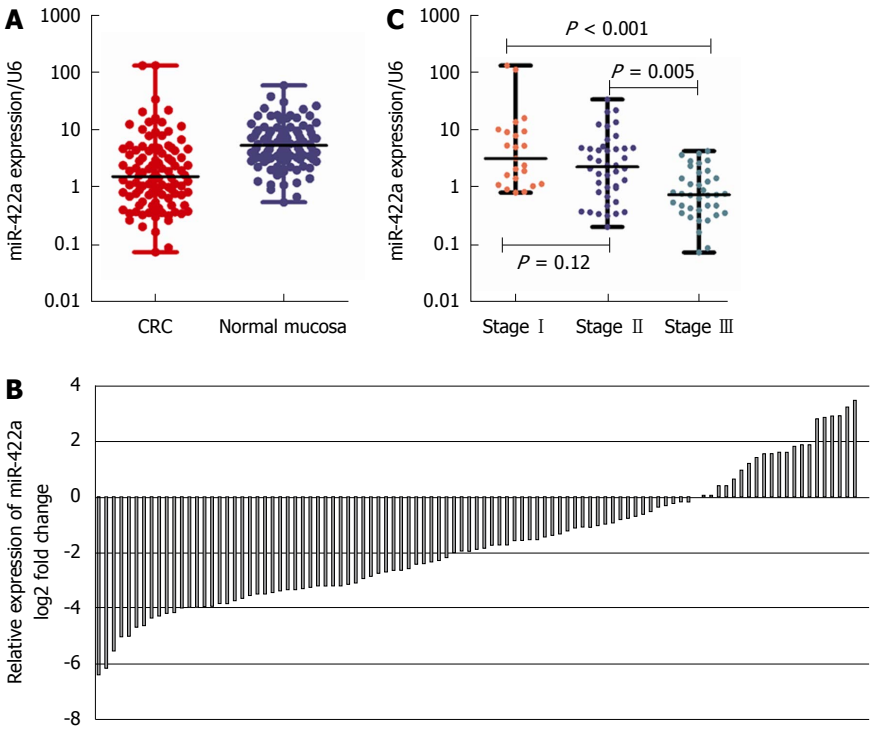

Figure 1 Expression of miR-422a in colorectal cancer and its association with TNM stage. A: Levels of miR-422a were determined in 102 colorectal cancer (CRC) tissues and their corresponding normal mucosa (NC); B: The fold changes of relative miR-422a level (CRC/NC) in each matched samples, with "< -1" defined as under-expression, "-1-1" as unchanged, and "> 1" as overexpression; C: Levels of miR-422a in stages Ⅰ/Ⅱ/Ⅲ CRC.

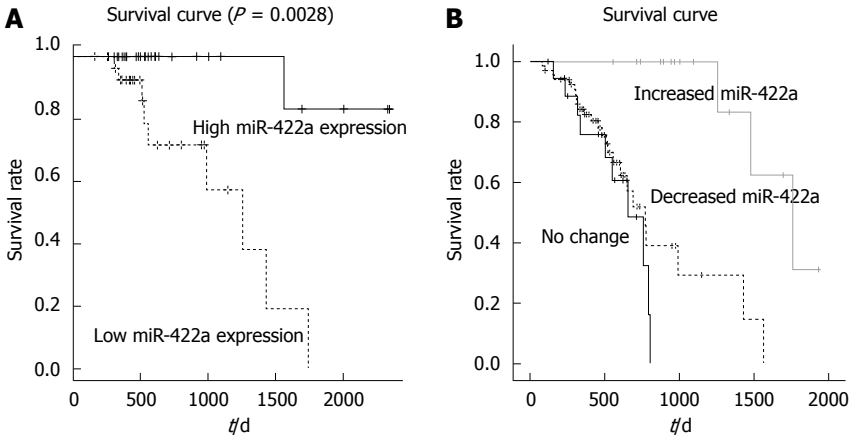

Figure 2 Kaplan-Meier curves for overall survival in 102 colorectal cancer patients based on miR-422a expression and the change of miR-422a expression compared to adjacent normal mucosa, respectively. Results showed that patients with low miR-422a expression had a significantly poorer prognosis than those with high miR-422a expression (A) (P = 0.0028), and patients with decreased miR-422a expression compared to adjacent normal mucosa had a significantly poorer prognosis than those with increased miR-422a expression (B) (P < 0.001).

> 0.05) (Figure 1C).

The association between miR-422a level and clinicopathological parameters of CRC patients is showed in Table 1. Our data showed that the level of miR-422a significantly correlated with local invasion (P = 0.004), lymph node metastasis (P = 0.002), and TNM stage (P < 0.001). However, there were no significant correlations between miR-422a level and gender, age, tumor location, differentiation or tumor size (P > 0.05).

miR-422a is an independent prognostic factor for overall survival in CRC patients

Our results demonstrated that 42 of 102 CRC patients died during the follow-up period. The 5-year overall survival rate was 58.8%. Patients were divided into high miR-422a expression and low miR-422a expression groups based on the median level. The prognosis was analyzed by Kaplan-Meier survival analysis, which revealed that patients with low miR-422a expression had a significantly poorer prognosis

Table 2 Univariate and multivariate analyses of overall survival in colorectal cancer patients

Variable	Category	Univariate analysis			Multivariate analysis		
		HR	95%CI	*P* value	HR	95%CI	*P* value
Gender	Male *vs* Female	0.943	0.493-1.872	0.810			
Age	≤ Median *vs* > Median	1.436	0.528-3.157	0.148			
Tumor location	Colon *vs* Rectum	0.862	0.484-1.706	0.603			
Tumor size	≤ 5 cm *vs* > 5 cm	1.093	0.568-2.194	0.873			
Differentiation	Well and moderate *vs* Poor	1.018	0.462-2.180	0.160	0.986	0.486-2.006	0.074
Local invasion	T1-T2 *vs* T3-T4	1.682	1.020-2.628	0.01[1]	1.460	0.746-2.632	0.482
Lymph node metastasis	Yes *vs* No	1.182	0.634-2.530	< 0.001[1]	1.262	0.584-2.680	0.306
TNM stage	Ⅰ and Ⅱ *vs* Ⅲ	2.738	1.509-4.265	< 0.001[1]	2.942	1.426-4.378	0.003[1]
MiR-422a level	Low *vs* High	0.306	0.108-0.763	0.001[1]	0.568	0.245-1.082	0.015[1]

[1]There was a significant difference between the two groups ($P < 0.05$).

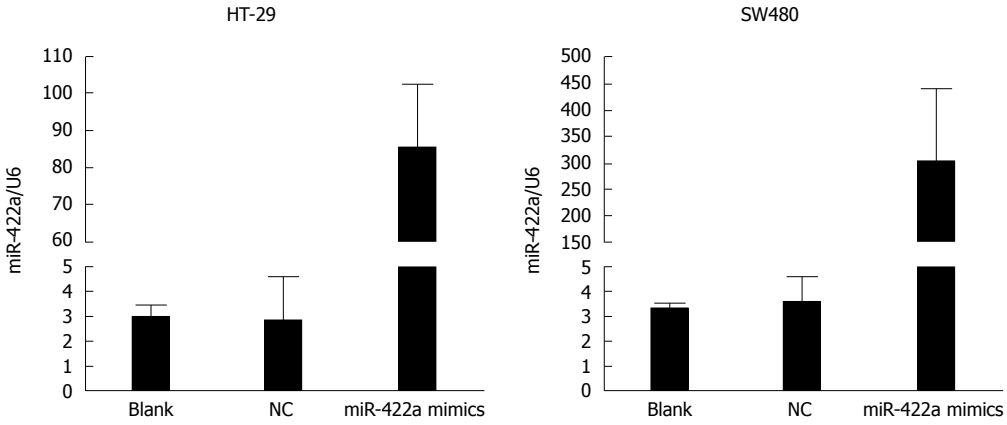

Figure 3 Transfection efficiency assays. The expression of miR-422a in HT-29 and SW480 cells was significantly increased after transfection with the mimics. All experiments were performed in triplicate.

than those with high miR-422a ($P = 0.0028$; Figure 2A). Furthermore, patients were divided into three groups (decreased, no change, and increased) based on the change of miR-422a expression compared to adjacent normal mucosa. Kaplan-Meier survival analysis showed that patients with decreased miR-422a expression had a significantly poorer prognosis than those with increased miR-422a expression ($P < 0.001$; Figure 2B). There was no significant difference between the decreased miR-422a group and no change group. In addition, Cox regression multivariate analysis was performed to determine whether miR-422a was an independent factor of overall survival in CRC patients. The analysis revealed that miR-422a expression (HR = 0.568, 95%CI: 0.245-1.082; $P = 0.015$) and clinical TNM stage (HR = 2.942, 95%CI: 1.426-4.378; $P = 0.003$) were independent prognostic factors for overall survival in CRC patients (Table 2).

Overexpression of miR-422a inhibits CRC cell proliferation

In our preliminary experiments, we detected the expression levels of miR-422a endogenously in several CRC cell lines, including HT-29, SW480, SW620 and HCT-116. The results showed that the levels of miR-422a were quite low in these CRC cells, and there were no significant differences in the expression level of miR-422a among these cell lines (data not shown). To measure the biological properties of miR-422a in CRC cells, miR-422a mimics were transiently transfected into HT-29 and SW480 cells, respectively. Subsequently, real-time RT-qPCR was performed, which showed high transfection efficiencies in both cell lines (Figure 3). The results of MTT assay showed that transfection of miR-422a mimics could significantly decrease cell number in SW480 and HT29 CRC cells ($P < 0.05$; Figure 4). It means that overexpression of miR-422a can inhibit the proliferation potential of SW480 and HT29 CRC cells.

Effect of miR-422a overexpression on cell migration and invasion

The transwell assays with and without Matrigel were performed to determine the effects of miR-422a on the migration and invasiveness of CRC cells. Overexpression of miR-422a significantly inhibited the migration of SW480 (P = 0.017; Figure 5A and B) and HT-29 cells ($P = 0.007$; Figure 5C and D). Furthermore, transwell assay with Matrigel showed that the invasiveness of these cells was significantly suppressed in cells transfected with miR-422a compared with miR control (Figure 6A-D).

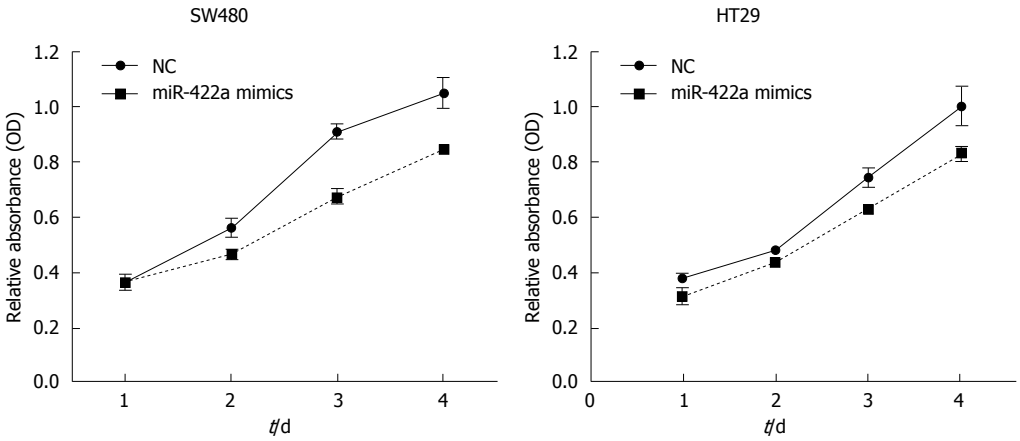

Figure 4 Overexpression of miR-422a inhibits colorectal cancer cell proliferation. Growth curves of miR-422a mimics and negative control mimics-transfected CRC cells were plotted after CCK-8 assay in SW480 and HT29 cells. All experiments were performed in triplicate. NC: Negative control.

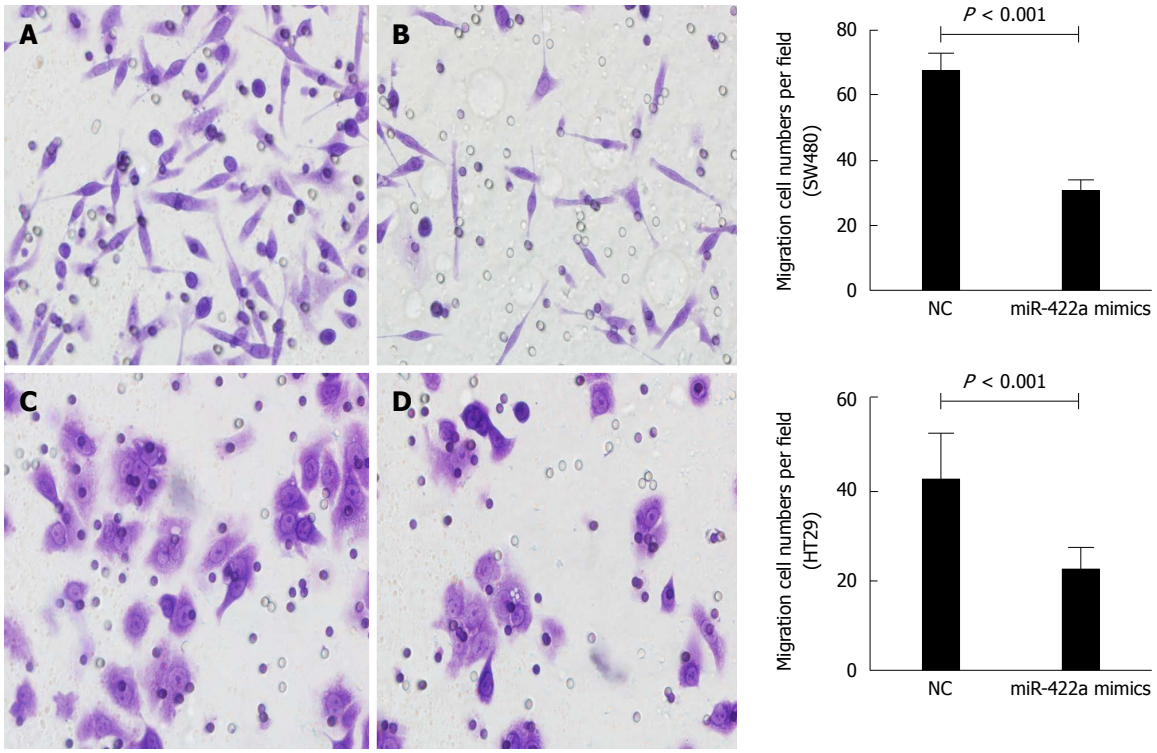

Figure 5 Effect of miR-422a overexpression on cell migration. Overexpression of miR-422a significantly inhibited the migration of SW480 (A and B) and HT29 (C and D) cells after transient transfection with miR-422a mimics. Stained cells were counted in five randomly selected fields under a light microscope. Representative photographs (left) and quantification (right) are shown. Magnification: × 400. All assays were performed in triplicate to derive the confidence intervals.

DISCUSSION

Tumor progression in CRC is a multi-step process involving a large number of genetic and epigenetic alterations[17-19]. Numerous studies have showed that miRNA target genes are involved in CRC tumorigenesis[6-8,20]. Currently, there is an ongoing effort to elucidate new deregulated miRNAs and their roles in CRC progression.

miR-422a is encoded by gene MIR422A on 15q22.31 (64, 163, 129-64, 163, 218bp). Recently, several studies have studied the functional role of miR-422a in osteosarcoma, osteoporosis, HIV infection, and other tumor types[21-25]. Our previous study demonstrated that serum miR-422a was significantly downregulated in CRC patients and is a potential biomarker with a high diagnostic accuracy[15]. However, the expression and role of miR-422a in CRC tissues, as well as its clinicopathological and prognostic value in CRC tumorigenesis remain undefined. In this study, our results demonstrated that miR-422a was down-regulated in CRC tissues compared with normal mucosa, which is consistent with a previous study[14]. We also found that the expression of miR-422a was significantly associated with lymph node metastasis and clinical stage in CRC

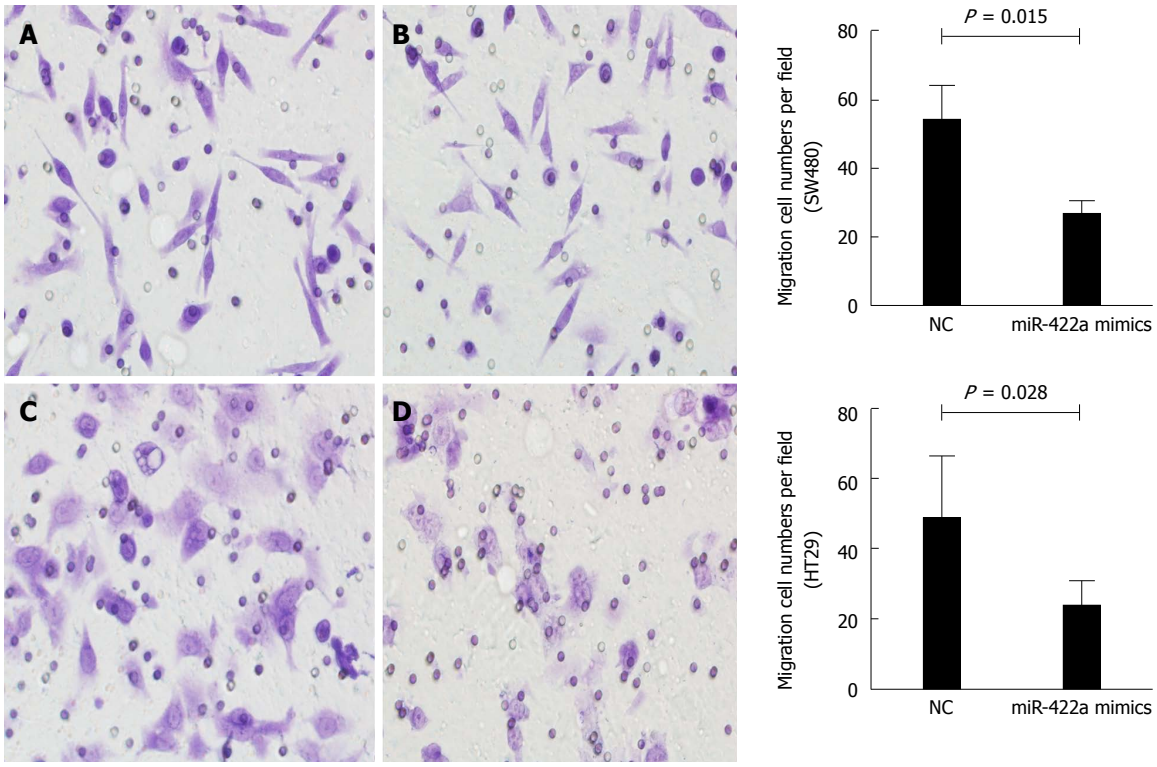

Figure 6 Effect of miR-422a overexpression on cell invasion. Overexpression of miR-422a significantly inhibited the invasiveness of in SW480 (A and B) and HT29 (C and D) cells after transient transfection with miR-422A mimics. Stained cells were counted in five randomly selected fields under a light microscope. Representative photographs (left) and quantification (right) are shown. Magnification: × 400. All assays were performed in triplicate to derive the confidence intervals.

patients, suggesting that down-regulation of miR-422a might participate in CRC progression. To further explore the value of miR-422a as a prognostic factor, we investigated the correlation between miR-422a and overall survival in CRC patients. Our results showed that patients with low expression of miR-422a and decreased miR-422a expression compared with normal mucosa had poorer survival, suggesting that miR-422a could serve as an independent prognostic factor in CRC patients. Overall, these results suggest that miR-422a plays a protective role against CRC and could be used to evaluate prognosis in CRC patients.

Until now, few studies have described the function of miR-422a[13,22,26]. A study has demonstrated that miR-422a and the mismatch repair protein Mutlα (MLH1) are involved in a feedback loop, leading to genome instability and tumorigenesis[13]. Another study showed that miR-422a significantly modulated the efficacy of IFN-α/RBV treatment *in vivo*. Exogenous IFN-α treatment led to decreased miR-422a and likely contributed to the IFN-mediated suppression of HIV-1, suggesting that restoring miR-422a treatment could be a potential therapeutic strategy for HIV-1 infection[26]. Moreover, overexpression of human telomerase reverse transcriptase (hTERT) has been associated with the invasion and metastasis of CRC cells. miR-138-5p and miR-422a were found to be hTERT-targeting miRNAs, potentially inhibiting hTERT expression[26]. Invasion and migration are required for tumor cells to spread from the primary site to lymph or blood vessels. In the present study, we found that miR-422a expression was associated with lymph node metastasis and overexpression of miR-422a inhibited CRC cell proliferation, migration and invasion. These results suggest that multiple signaling pathways regulating different aspects of tumorigenesis may be regulated by miR-422a expression, suggesting that miR-422a may be a new therapeutic target to repress cancer progression.

COMMENTS

Background

A growing number of studies have shown that deregulated miRNAs play critical roles in tumorigenesis and progression of colorectal cancer (CRC). Nevertheless, an ongoing effort to elucidate new deregulated miRNAs and their roles in CRC is still urgently needed. Several studies have demonstrated the importance of miR-422a in different types of human diseases. In our previous study, miR-422a was found to be down-regulated in serum and could be used as a potential biomarker for CRC. Therefore, it is necessary to further determine the expression of miR-422a in CRC tissues and to explore its clinicopathological, prognostic value and role in CRC carcinogenesis.

Research frontiers

Several studies have determined the importance of miR-422a in human diseases such as cancer, multiple sclerosis, and postmenopausal osteoporosis. miR-422a could inhibit signaling pathways regulating tumor cell proliferation in osteosarcoma. It was also shown that miR-422a targeted key mismatch repair protein (MutLα) by suppressing MLH1 expression, resulting in genome instability and tumorigenesis. Previously, we identified a serum 4-miRNA panel that included miR-19a-3p, miR-92a-3p, miR-223-3p, and miR-

422a. This miRNA panel served as biomarkers for early diagnosis of colorectal adenocarcinoma.

Innovations and breakthroughs

The present study indicated that down-regulation of miR-422a might serve as an independent prognosis factor in CRC. miR-422a functions as a tumor suppressor and regulates progression of CRC.

Applications

By understanding the differential expression of miR-422a in CRC patients and its relationship with clinicopathological characteristics, prognosis and *in vitro* function, the present study may provide a new prognostic factor of CRC and further reveal the mechanism of miR-422a participating in CRC carcinogenesis.

Terminology

MicroRNAs are a class of short non-coding RNAs that regulate up to 30% of human genes and play a pivotal role in various cellular processes. A growing number of miRNAs have been implicated in the initiation and progression of tumors including miR-422a. miR-422a is encoded by gene *MIR422A* on 15q22.31 (64,163,129-64,163,218bp), which has been found to be important in human diseases such as cancer, multiple sclerosis, and postmenopausal osteoporosis.

Peer-review

The authors presented a study analyzing the prognostic value and function of miR-422a in CRC carcinogenesis. The results demonstrated that miR-422a was dramatically reduced in CRC tissues and significantly correlated with local invasion and lymph node metastasis. miR-422a expression was an independent prognostic factor for overall survival. *In vitro* experiments showed that overexpression of miR-422a inhibited the proliferation, migration, and invasion of SW480 and HT-29 cells. These data indicated that miR-422a might serve as an independent prognosis factor and regulate progression of CRC by functioning as a tumor suppressor.

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ORIGINAL ARTICLE

Retrospective Study

Colostomy is a simple and effective procedure for severe chronic radiation proctitis

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Author contributions: Yuan ZX and Ma TH contributed equally to this work; Yuan ZX, Ma TH and Wang L conceived and designed the study; Yuan ZX, Zhong QH, Yu XH, and Qin QY and Wang L conducted the experiments; Yuan ZX, Ma TH, Wang HM and Wang JP analyzed and interpreted the data; Yuan ZX, Ma TH and Wang L wrote and revised the manuscript; all authors approved the final version to be published.

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Institutional review board statement: The study was approved by the Ethical Committee of the Sixth Affiliated Hospital of Sun Yat-Sen University and fulfilled the guidelines of the local responsible governmental agency.

Informed consent statement: Informed consent was waived due to the retrospective nature of this study.

Conflict-of-interest statement: The authors declare no conflicts of interests related to the publication of this study.

Data sharing statement: No additional data was available.

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Abstract

AIM: To assess the efficacy and safety of diverting colostomy in treating severe hemorrhagic chronic radiation proctitis (CRP).

METHODS: Patients with severe hemorrhagic CRP who were admitted from 2008 to 2014 were enrolled into this study. All CRP patients were diagnosed by a combination of pelvic radiation history, clinical rectal bleeding, and endoscopic findings. Inclusion criteria were CRP patients with refractory bleeding with moderate to severe anemia with a hemoglobin level < 90 g/L. The study group included patients who were treated by diverting colostomy, while the control group included patients who received conservative treatment. The remission of bleeding was defined as complete cessation or only occasional bleeding that needed no further treatment. The primary outcome was bleeding remission at 6 mo after treatment. Quality of life before

treatment and at follow-up was evaluated according to EORTC QLQ C30. Severe CRP complications were recorded during follow-up.

RESULTS: Forty-seven consecutive patients were enrolled, including 22 in the colostomy group and 27 in the conservative treatment group. When compared to conservative treatment, colostomy obtained a higher rate of bleeding remission (94% *vs* 12%), especially in control of transfusion-dependent bleeding (100% *vs* 0%), and offered a better control of refractory perianal pain (100% *vs* 0%), and a lower score of bleeding ($P < 0.001$) at 6 mo after treatment. At 1 year after treatment, colostomy achieved better remission of both moderate bleeding (100% *vs* 21.5%, $P = 0.002$) and severe bleeding (100% *vs* 0%, $P < 0.001$), obtained a lower score of bleeding (0.8 *vs* 2.0, $P < 0.001$), and achieved obvious elevated hemoglobin levels ($P = 0.003$), when compared to the conservative treatment group. The quality of life dramatically improved after colostomy, which included global health, function, and symptoms, but it was not improved in the control group. Pathological evaluation after colostomy found diffused chronic inflammation cells, and massive fibrosis collagen depositions under the rectal wall, which revealed potential fibrosis formation.

CONCLUSION: Diverting colostomy is a simple, effective and safe procedure for severe hemorrhagic CRP. Colostomy can improve quality of life and reduce serious complications secondary to radiotherapy.

Key words: Chronic radiation proctitis; Rectal bleeding; Diverting colostomy; Quality of life; Serious complication

Core tip: The study describes the efficacy and safety of diverting colostomy in treating severe hemorrhagic chronic radiation proctitis. The procedure focuses on improving the severe refractory bleeding and reducing severe complications. The advantages of diverting colostomy are as follows: it acts effectively and rapidly in controlling severe bleeding that does not respond to conservative treatment; it is a simple procedure that can be conducted in many medical centers; and it can improve quality of life dramatically and reduce serious complications that occur secondary to radiotherapy.

Yuan ZX, Ma TH, Wang HM, Zhong QH, Yu XH, Qin QY, Wang JP, Wang L. Colostomy is a simple and effective procedure for severe chronic radiation proctitis. *World J Gastroenterol* 2016; 22(24): 5598-5608 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i24/5598.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i24.5598

INTRODUCTION

Chronic radiation proctitis (CRP) is a common complication after radiotherapy of pelvic malignancies, accounting for 5%-20% of cases[1]. The onset of RP can be delayed for several months to years after radiotherapy. CRP develops as a result of ischemic lesions due to obliterative endarteritis and progressive fibrosis[2,3]. Rectal bleeding is the most common symptom, which accounts for > 80% of CRP patients[4]. Acute and mild CRP is usually self-limiting and easy to manage, but moderate to severe CRP is difficult to treat; especially those cases requiring blood transfusions and that are life threatening[1,5].

Various treatment modalities have been tried. Medical agents include topical sucralfate, steroids[6], sulfasalazine[7], metronidazole[8], rebamipide[9] and short-chain fatty acid[10]. Other treatment options include topical formalin[11,12], endoscopic argon plasma coagulation (APC)[13], laser therapy[14], and hyperbaric oxygen therapy[15]. However, most of these treatments are only useful for mild to moderate bleeding, and severe and refractory bleeding is still problematic[16]. Furthermore, endoscopic treatments can bring severe side effects and multiple treatment sessions are needed for severe CRP[17]. In addition, accompanying symptoms such as intractable perianal pain, urgency and tenesmus in CRP are usually hard to manage.

Diverting colostomy has been reported previously, mainly for severe CRP complications[18,19]. Colostomy can reduce the irritation injury of fecal stream to the irradiated tissues and thus decrease rectal bleeding. However, unlike formalin or APC, colostomy is now not widely used. The issue of colostomy is not well studied to date. To the best of our knowledge, no study has compared diverting colostomy to conservative treatment. The aim of this study was to assess the efficacy and safety of diverting colostomy in severe CRP. The indications, quality of life, severe CRP complications, and stoma reversals after colostomy were also investigated, when compared to conservative treatment.

MATERIALS AND METHODS

Patients and ethical statements

Hemorrhagic CRP patients who were treated at the Sixth Affiliated Hospital of Sun Yat-Sen University (SYSU) from March 2008 to October 2014 were retrospectively enrolled in this study. Electronic files and medical records were both carefully collected to extract clinicopathological data. This study was approved by the Ethical Committee of the Sixth Affiliated Hospital of SYSU and the study was conducted in accordance with the provisions of the World Medical Association's Declaration of Helsinki in 1995 (revised in Tokyo, 2004). Due to the nature of

Table 1 Modified subjective objective management analysis system to assess the severity of bleeding in radiation proctitis

Grade	Bleeding	Severity	Anemia (Hb, g/L)
1	Mild bleeding	Occasionally or occult	Mild anemia (Hb: ≥ 90 g/L)
2	Moderate bleeding	Persistent	Moderate anemia (Hb: 70-90 g/L)
3	Severe bleeding	Gross	Severe anemia, transfusion needed (Hb: < 70 g/L)

Hb: Hemoglobin.

the retrospective study, informed consent was waived.

Inclusion and exclusion criteria

Inclusion criteria were CRP patients with refractory bleeding with moderate to severe anemia with a hemoglobin level < 90 g/L. Refractory bleeding was defined as no response to conservative treatment. Patients who were treated with diverting stomas were enrolled in the study group, while those who continued to non-surgical treatment were enrolled in the control group. Patients with tumor relapses, loss to follow-up, or who underwent rectal resection with preventive colostomy were excluded.

Diagnosis, scores and definitions

All patients were diagnosed by combination of pelvic radiation history, clinical rectal bleeding, and endoscopic findings of injured rectal mucosa. Flexible colonoscopy was performed in all patients to rule out other causes of bleeding, such as recurrent tumors, inflammatory bowel disease and anal benign hemorrhagic diseases. The Vienna Rectoscopy Score[20] system was used to assess endoscopic severity.

Current scores to evaluate the severity of bleeding included Common Terminology Criteria for Adverse Events[21], Radiation Therapy Oncology Group/European Organization for Research and Treatment of Cancer (EORTC) score[22]. However, most of them are based on subjective complaints of patients, instead of accurate laboratory tests. Because the severity of bleeding was mainly reflected by a decrease in hemoglobin level, we designed a modified Subjective Objective Management Analysis (SOMA) system reported in a previous study[23], to assess the severity of bleeding, included both subjective complaints of bleeding and objective hemoglobin level. All patients were scored by the system (Table 1). The remission of bleeding was defined as complete cessation of bleeding or only occasional bleeding that needed no further treatment. Failure in the conservative treatment group was defined as no improvement or even worse bleeding and decreased hemoglobin level 6 mo after treatment.

Indications of diverting colostomy

All patients, except those with fistulas, were initially treated with medical agent enemas including almagate (one mucosa-protector like sucralfate), corticosteroids, and metronidazole. Topical formalin (details listed in our previous study)[24] or endoscopic APC were suggested when they experienced recurrent bleeding. As for refractory and transfusion-dependent CRP after these conservative measures, physicians suggested a diverting stoma. If patients refused a colostomy and demanded to continue conservative treatment, they were enrolled in the control group. Other indications of diverting colostomy were as follow: (1) fistula, perforation or stricture; and (2) deep ulcer with refractory perianal pain.

Diverting loop colostomies were conducted under general anesthesia in the operating room. The transverse colon was pulled out through a small incision, then a soft catheter of a stent was inserted to prevent stoma retraction, and a double-cavity stoma of the transverse colon was then created. The catheter was removed postoperatively. Classical images of a double-cavity colostomy and a "gunsight" of stoma closure were shown (Figure 1). This technique of stoma closure can simplify wound care, decrease surgical site infection, and give a neat cosmetic result[25,26].

Follow-up

Follow-up was scheduled through outpatient visits or telephone questionnaires at 6 mo and 1 year after treatment. The quality of life before treatment and at follow-up was evaluated according to EORTC QLQ C30[27]. The primary outcome was the remission rate of bleeding at 6 mo after treatment. The secondary outcomes included hemoglobin level, remission rate of bleeding at 1 year after treatment, quality of life, stoma-related complications, severe CRP complications, and stoma reversal rate.

Statistical analysis

Comparisons of characteristics were made by Student's *t* test analysis for continuous variables. For categorical variables, the χ^2 test was used. Fisher's exact test was adopted when appropriate. For non-parameter variables, the Wilcoxon rank sum test was used. All statistical analyses were performed by SPSS version 17.0 (Chicago, IL, United States). $P < 0.05$ (two-tails) was considered to be statistically significant.

RESULTS

Demographics and characteristics

A total of 47 patients were analyzed. Twenty-two (46.8%) were treated by diverting colostomy, and 27 (53.2%) were managed by conservative treatment (Figure 2). Forty-three (91.5%) were women, and 40 (85.1%) primary malignancies were cervical cancer. Cumulative radiation dosage of one patient was about 80 Gy, which included the radiation for both sites

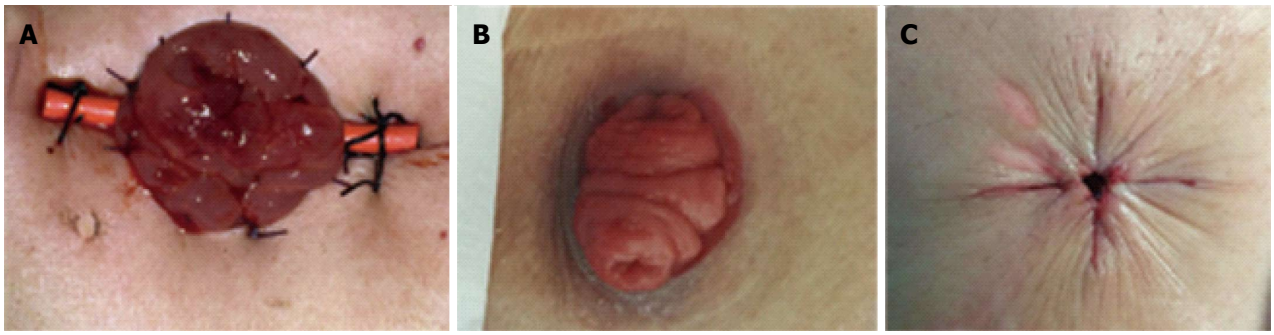

Figure 1 Classic representative images of a double-cavity colostomy. A: A double-cavity colostomy with a soft catheter as a stent; B: A colostomy after during follow-up; C: A "gunsight" skin incision and closure for stoma reversal.

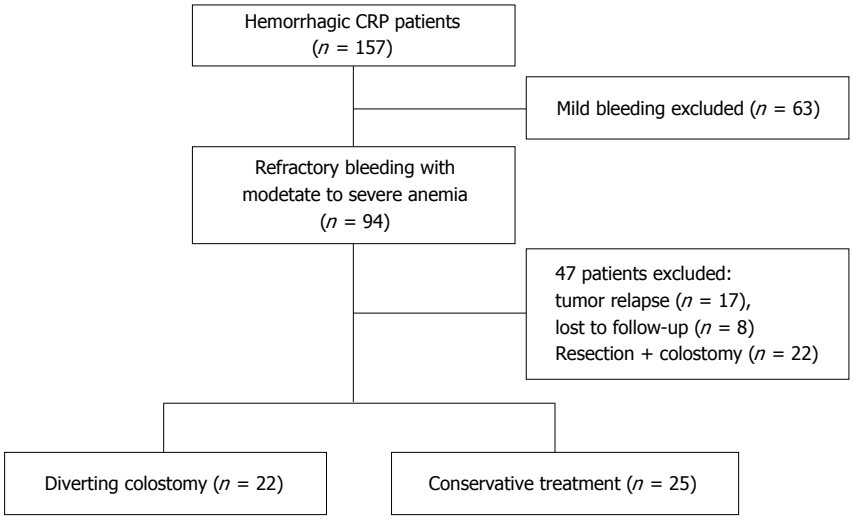

Figure 2 Flow chart of patient selection.

of primary malignancy and invasive lymph nodes. The detailed radiotherapy for those patients with gynecological cancers, especially cervical cancer, was 25 rounds (2 Gy/round) of external beam radiation and five or six episodes (6 Gy/episode) of intra-cavity brachytherapy. Patients with prostate or rectal cancers received only external beam radiation. When comparing demographics prior to treatment between the two groups, there were no significant differences in age, gender, type of primary malignancy, cumulative radiation dosage, latency period, duration from treatment to end of radiotherapy, duration of bleeding, albumin level, body mass index (BMI), concomitant radiation uropathy, radiation enteritis, and associated risk factors of CRP such as previous history of abdominal surgery, diabetes mellitus and hypertension (Table 2). Thus, these above characteristics were comparable between the two groups. However, the colostomy group had a higher score of bleeding (2.7 *vs* 2.0, $P < 0.001$) and a lower hemoglobin level (60.8 g/L *vs* 88.2 g/L, $P < 0.001$), when compared to the conservative treatment group, respectively. These results indicated that the colostomy group had more serious bleeding before treatment (Table 2).

Treatment

The indications for colostomy were as follows: (1) severe bleeding in eight (36.4%) cases; (2) fistulas in 11 (50%), including nine (40.9%) rectovaginal fistulas and two (9.1%) sigmoid-vesical-vaginal fistulas; (3) deep ulcer + refractory perianal pain in two (9.1%) cases; and (4) severe bleeding + deep ulcer + anal stricture in one (4.5%) case. Among these 11 patients with fistulas, five also had concomitant severe bleeding. Among these nine recto-vaginal fistulas, one had concomitant recto-urethral fistula, and another had concomitant recto-vesical fistula and small bowel fistula.

In the conservative treatment group, seven patients received topical formalin irrigation and one received APC treatment after enrollment. All eight (32%) cases transiently obtained bleeding remission, but only two (25%) obtained long-term remission of bleeding. The other six patients experienced recurrent bleeding and developed severe anemia. Repeat topical formalin achieved only limited efficacy in these patients with severe anemia (average 2 sessions of formalin at 2-4-wk intervals). The remaining 17 patients refused formalin treatment, and thus continued retention

Table 2 Comparisons of patient demographics between the colostomy group and conservative group before treatment *n* (%)

Characteristics	Diverting colostomy (*n* = 22)	Conservative treatment (*n* = 25)	*P* value
Age (mean ± SD)	60.1 ± 2.2	60.2 ± 2.4	0.964
Gender (female/male)	20/2	23/2	1.000[1]
Primary malignancy			0.822[2]
Cervical cancer	19 (86.4)	21 (84)	
Endometrial cancer	2 (9.1)	2 (8)	
Rectal cancer	1 (4.5)	1 (4)	
Prostate cancer	0 (0)	1 (4)	
Cumulative radiation dosage (Gy), mean ± SD	80.5 ± 17.3	83.6 ± 20.5	0.842[3]
Latency period (mo), mean ± SD	8.3 ± 0.8	7.2 ± 1.1	0.252[3]
Duration from treatment to end of radiotherapy (mo), mean ± SD	16.3 ± 1.3	14.8 ± 1.6	0.277[3]
Duration of bleeding (month), mean ± SD	7.9 ± 1.0	8.0 ± 1.8	0.466[1]
Score of bleeding, mean ± SD	2.7 ± 0.5	2.0 ± 0.5	< 0.001[3]
Mean hemoglobin (g/L), mean ± SD	60.8 ± 18.1	88.2 ± 19.3	< 0.001
Alb (g/L), (≤ 35/> 35)	6/16	3/22	0.339[1]
BMI (kg/m^2), (≤ 17.5/> 17.5)	3/19	2/23	0.880[1]
Concomitant radiation uropathy	8 (36.4)	6 (24)	0.355
Radiation enteritis	5 (22.7)	2 (8)	0.315[1]
Previous abdominal surgery	6 (27.3)	11 (44)	0.234
Diabetes mellitus	2 (9.1)	3 (12)	1.000[1]
Hypertension	6 (27.3)	7 (28)	0.956

[1]χ^2 test; [2]Fisher exact test; [3]Wilcoxon rank-sum test. BMI: Body mass index; Alb: Albumin.

Table 3 Bleeding remissions in severe radiation proctitis after treatment

Variables	Diverting Colostomy (*n* = 22)	Conservative treatment (*n* = 25)	*P* value
6 mo after treatment			
Remission of bleeding	17/18 (94%)	3/25 (12%)	< 0.001[1]
Remission of refractory perianal pain	8/8 (100%)	0/6 (0%)	< 0.001[2]
Score of bleeding, mean ± SD	1.1 ± 0.5	2.2 ± 0.7	< 0.001[3]
Elevated Hb, mean ± SD	34.1 ± 18.2	-12.3 ± 9.1[4]	< 0.001
Remission of moderate bleeding	8/8 (100%)	6/19 (21.5%)	0.002[2]
Remission of severe bleeding	11/11 (100%)	0/5 (0)	< 0.001[2]
Score of bleeding, mean ± SD	0.8 ± 0.5	2.0 ± 0.9	< 0.001[3]
Post-treatment recto-vaginal fistula	0/22	3/25 (12%)	0.237[2]
Elevated Hb, mean ± SD	40.3 ± 19.3	-1.9 ± 32.5[4]	0.003

[1]χ^2 test; [2]Fisher exact test; [3]Wilcoxon rank-sum test; [4]Represents decreased level.

enemas and transfusions when needed.

Outcomes

During a mean 22 (range: 6-77) mo of follow-up, eight (17%) patients died. The cause of death was recurrent malignancy in seven cases. The other one died of bladder perforation and sepsis that occurred secondary to radiation recto-vesical-vaginal perforation. At 6 mo after treatment, colostomy offered higher remission of bleeding (94% *vs* 12%, $P < 0.001$), higher remission of refractory perianal pain (100% *vs* 0%, $P < 0.001$), decreased scores of bleeding (1.1 *vs* 2.2, $P < 0.001$), and obvious increased hemoglobin levels (34.1 g/dL *vs* -12.3 g/dL, $P < 0.001$), compared to the conservative treatment group. At 1 year after treatment, colostomy achieved still higher remission of both moderate bleeding (100% *vs* 21.5%, $P = 0.002$) and severe bleeding (100% *vs* 0%, $P < 0.001$), acquired lower score of bleeding (0.8 *vs* 2.0, $P < 0.001$), and obviously elevated hemoglobin levels (40.3 g/dL *vs* -1.9 g/dL, $P = 0.003$), than those cases in the conservative treatment group. In addition, three recto-vaginal fistulas were found in the conservative treatment group during follow-up, but no new fistula occurred after the operation in the colostomy group. Patients who did not have bleeding remission continued conservative treatment at home (Table 3).

Stoma closure and complications

Of the eight patients who received colostomy to control severe bleeding, three (37.5%) underwent stoma closure (2 cases at 9 mo and 1 at 10 mo after colostomy). All three had no bleeding and remained well after stoma reversal. Of the remaining five, all obtained bleeding remission and improved hemoglobin levels. However, among these five, one had grade IV New York Heart Association heart failure and could not risk stoma closure. Four were unsuitable for closure

Table 4 Quality of life between diversion group and conservative group in CRP patients by EORTC QLQ-C30 scale

QLQ-C30 scale	Ref. (Normal German population)	Diverting colostomy group (n = 18), mean (SD)				Conservative treatment group (n = 23), mean (SD)			
		Pre-treatment	Follow-up	Δ(FU)-Pre[1]	Significance[2]	Pre-treatment	Follow-up	Δ(FU)-Pre	Significance[2]
Global health	63.2	23.1 (15.1)	64.8 (13.8)	41.7	< 0.001	47.1 (21.5)	62.3 (25.0)	15.2	0.033
Physical function	82.6	50.7 (17.8)	77.8 (16.6)	27.1	< 0.001	78.0 (22.7)	78.6 (26.1)	0.6	0.856
Role function	75.0	34.3 (23.9)	75.9 (27.3)	41.6	< 0.001	77.5 (24.9)	77.5 (29.1)	0.0	0.775
Emotional function	62.2	46.3 (27.4)	73.6 (27.0)	27.3	0.001	75.7 (17.6)	80.8 (23.8)	5.1	0.384
Cognition function	81.3	92.6 (12.7)	93.5 (9.8)	0.9	0.581	94.2 (15.6)	95.7 (9.0)	1.5	0.798
Social function	78.4	43.5 (28.9)	65.7 (31.7)	22.2	0.004	91.3 (20.6)	89.1 (21.1)	-2.2	0.916
Fatigue	34.1	72.8 (12.9)	36.4 (25.9)	-36.4	< 0.001	26.6 (24.1)	23.2 (27.2)	-3.4	0.695
Nausea/vomiting	5.7	4.6 (15.5)	1.9 (7.6)	-2.7	0.109	5.1 (15.4)	6.5 (16.5)	1.4	0.655
Pain	33.1	44.4 (28.3)	14.8 (22.8)	-29.6	0.001	14.5 (21.5)	11.6 (18.4)	-2.9	0.481
Dyspnea	18.8	42.6 (31.0)	18.5 (22.8)	-24.1	0.003	14.5 (19.7)	8.7 (18.0)	-5.8	0.210
Insomnia	38.5	48.1 (35.5)	29.6 (31.2)	-18.5	0.026	21.7 (21.6)	26.1 (31.7)	4.4	0.287
Appetite loss	9.4	22.2 (27.2)	13.0 (19.7)	-9.2	0.125	10.1 (25.5)	8.7 (18.0)	-1.4	0.785
Constipation	9.1	9.3 (14.9)	3.7 (10.5)	-5.6	0.066	8.7 (20.6)	7.2 (22.4)	-1.5	0.414
Diarrhea	9.2	33.3 (33.3)	22.2 (24.8)	-11.1	0.018	11.6 (23.8)	2.9 (9.6)	-8.7	0.078
Financial difficulties	17.1	59.3 (26.2)	50.0 (31.9)	-9.3	0.211	26.1 (31.7)	24.6 (30.5)	-1.5	0.595

[1]Wilcoxon rank-sum test; [2]Point (follow-up) - point (pre-treatment).

because of erythema and telangiectasia at 6 mo after colostomy, and two of these four had not yet reached 1 year follow-up to assess the lesion by colonoscopy.

Stoma complications were found in seven (31.8%) cases, which contained six stoma prolapses and one stoma stricture. Of these six stoma prolapses, four were managed with conservative measures by manual repositions (Grade Ⅱ by Clavien-Dindo classification[28]), and two required stoma rebuilding (Grade Ⅲ). One stoma stricture occurred in a stoma of the descending colon, instead of the transverse colon, and stoma stricture was managed by finger dilatation (Grade Ⅱ).

Quality of life

The quality of life was evaluated in 41 (87.2%) patients by the EORTC QLQ-C30 questionnaires. Because there were no similar reports in the Chinese population, the values were referred to the normal German population. Osoba *et al*[29] suggested that a difference of ⩾ 20 points in global health was considered to be clinically relevant. In this study, when compared to pre-treatment, diverting colostomy improved quality of life, including improved global health (difference = 42, $P < 0.001$), improved functions like physical function ($P < 0.001$), role function ($P < 0.001$), emotional function ($P < 0.001$), social function ($P < 0.001$), and improved symptoms like fatigue ($P < 0.001$), pain ($P = 0.001$), dyspnea ($P = 0.003$), insomnia ($P = 0.026$), and diarrhea ($P = 0.018$). However, conservative treatment did not significantly improve quality of life at follow-up compared with before treatment (Table 4).

Endoscopic and pathological features

Classical endoscopic images prior to colostomy and at stoma closure from three patients with stoma closures were collected (Figure 3). After a mean 9.3 (range: 9-10) mo after colostomy, endoscopic lesions of active bleeding, multiple confluent telangiectasia, congested mucosa, or even ulcer were greatly improved and reached the criteria of stoma closure.

Pathological evaluation of endoscopic biopsy from rectal lesions was conducted at 31 mo after colostomy in Case 3. This patient obtained complete remission of bleeding and the biopsy sites were fully healed according to endoscopic observation at follow-up. Pathologically, diffuse chronic inflammatory cells were observed in the mucosa and sub-mucosa layers. In addition, massive fibrotic collagen depositions were found in the sub-mucosa layer, which revealed fibrosis formation (Figure 4).

DISCUSSION

Endoscopic treatments, such as topical formalin or APC, are extensively used for mild to moderate hemorrhagic CRP worldwide[14,16]. However, these treatments show only limited long-term efficacy for severe CRP[17]. It is also unclear how many patients have received these treatments for serious transfusion-dependent bleeding in previous studies[17]. Meanwhile, serious complications can be caused by these endoscopic treatments[5,17]. Our previous study also suggested that topical formalin should not be applied in CRP with ulcer because of the risk of fistula[24]. In the present study, patients in the conservative treatment group received more topical formalin or APC treatment and developed more fistulas later, than patients in the colostomy group. Therefore, we suggest topical formalin or APC should be selected cautiously for patients with severe CRP. Recently, radiofrequency ablation (RFA) in treating CRP has been introduced, with improvement in hemoglobin level and decrease in clinical symptoms[30,31]. Most RFA studies are based

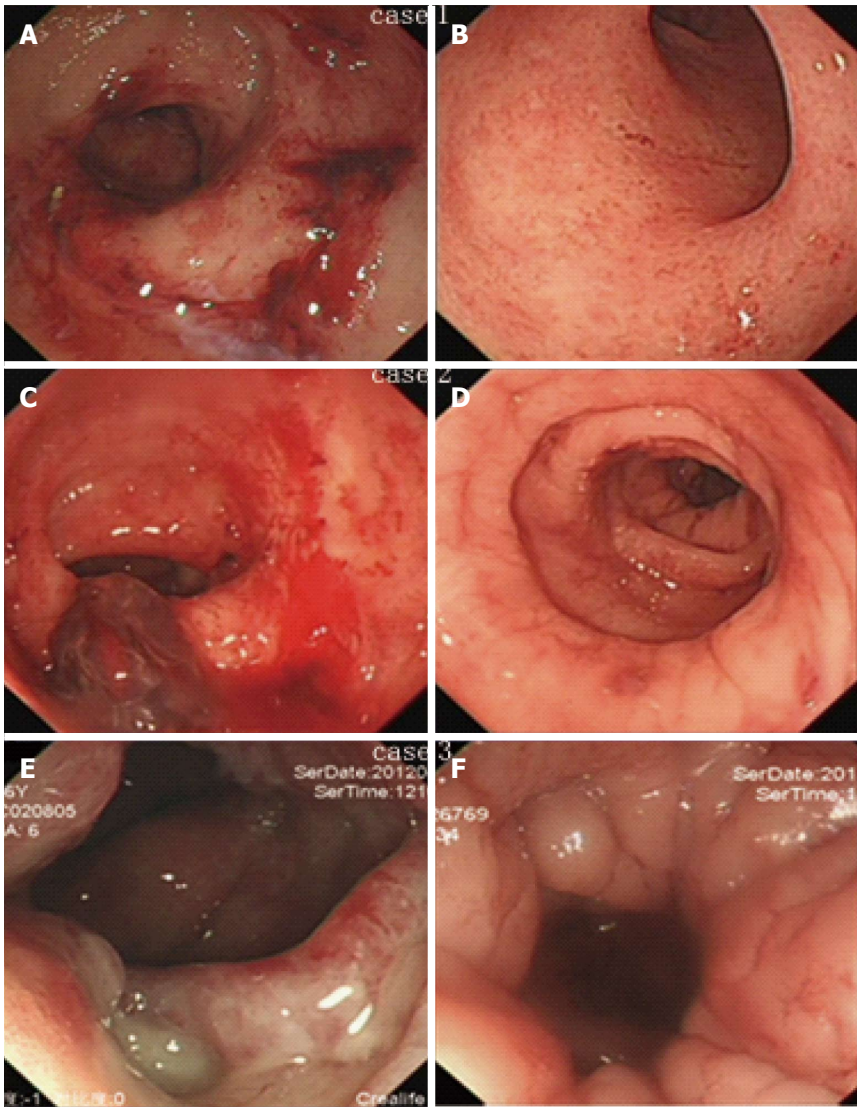

Figure 3 Classical endoscopic images before colostomy and at stoma reversal. Severe active bleeding (A and C), or confluent telangiectasia, edema and ulcer (E) were observed in cases 1-3 before colostomy, while these lesions was greatly improved at stoma reversal (B, D, F).

on retrospective case series without controls and current data are scare, therefore, prospective trials of RFA should be conducted in the future to validate its efficacy and application in severe CRP.

Surgical intervention is often the last resort for severe CRP[3,19]. Rectal resection is controversial, because it is difficult to perform a safe anastomosis in the radiation-injured tissue, and high risks of anastomotic leak and death from postoperative peritonitis are reported[19]. Therefore, a simple and safe procedure to save life and relive symptoms is mandatory. Theoretically, diverting colostomy can reduce bacterial contamination and decrease irritation injury by fecal stream, and colostomy can gain time to subside any radiation reaction to protect injured tissue[32]. Thus, severe bleeding and refractory perianal pain can be controlled. Colostomy can also accelerate the course of fibrosis and relieve severe proctitis rapidly, which may prevent deep ulcers progressing to fistulas. In our recent study[33], we reported typical histopathological features of CRP: telangiectasia, abnormal hyaline-like wall vessels and sporadic radiation fibrocytes in the submucosal layer. In this study, consistently, massive fibrotic collagen depositions were observed in irradiated tissue after colostomy. Collectively, the nature of CRP is a progressive fibrosis course[7]. The efficacy and safety of colostomy have been reported in previous studies[18,19,34]. However, most previous studies did not contain controls, which could not discriminate the efficacy of interventions from the self-remission course. To the best of our knowledge, no previous study has reported quality of life after colostomy. It is also not clear whether colostomy can reduce serious CRP complications.

In this study, diverting colostomy resolved most cases of severe bleeding. No recurrent bleeding and no colostomy-related death were observed. Colostomy

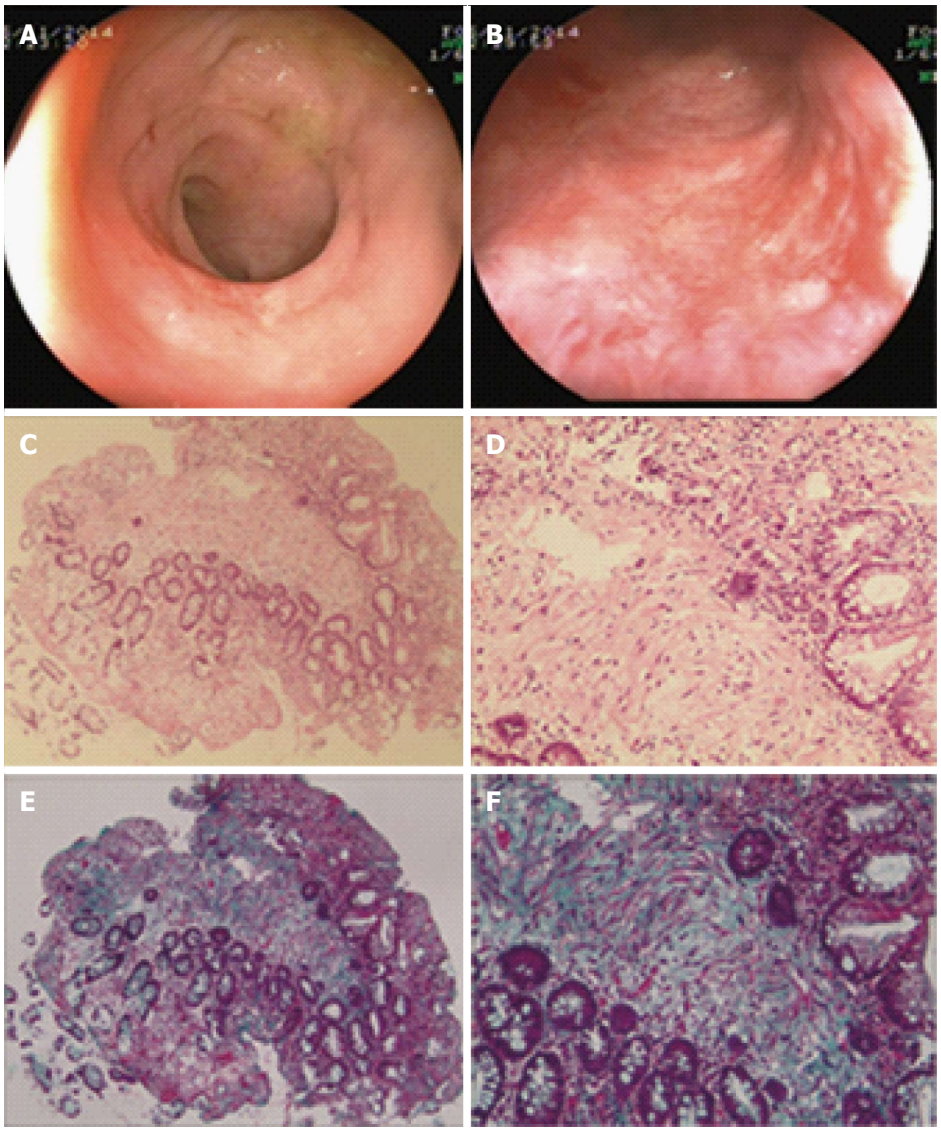

Figure 4 Histo-pathological features after colostomy in case 3. Complete remission of confluent telangiectasia, edema and ulcer were observed 31 mo after colostomy (A and B). Diffused chronic inflammatory cells and rebuilt of mucosal integrity were seen in hematoxylin and eosin-stained slides (C: × 100; D: × 400). Massive fibrotic collagen depositions were observed in the sub-mucosa layer, with green color by Masson staining (E: × 100; F: × 400).

can also decrease serious complications, including remission of long-term perianal pain, transfusion-dependent bleeding and fistula. Deep ulcer with severe bleeding is a contraindication to endoscopic treatment, because it is easy to progress to fistula, due to the poor-healing capacity and impaired blood supply of friable intestinal wall[17,19]. We have previously tried topical revision and skin flap transplantation for some CRP fistulas. However, the efficacy was limited and new fistulas can occur rapidly, due to poor healing capacity of irradiated mucosa and bacterial infection from the fecal stream, which leads to treatment failure. In this study, two patients with deep ulcer and severe bleeding were successfully controlled by colostomy, and no fistula was found at follow-up.

Transverse colostomy is preferred because it provides a greater blood supply by preserving superior rectal and left marginal vessels, and provides more options for later possible recto-sigmoid resection than sigmoid colostomy does, and transverse colostomy is easier to be closed and is more effective[32,34]. In addition, the recto-sigmoid colon is expected to receive a higher radiation dose for pelvic malignancy, while the transverse colon receives the least radiation and damage, because it is located far from pelvic tumors, and thus causes the fewest complications[19,32,35]. Loop ileostomy is not widely used because of the risk of high-volume fluid discharge. Colostomy-related complications were reported, ranging from 21.8% to 40%[19,35]. Consistent with previous studies, colostomy-related complications in this study were 31.8%, including six (85%) cases of Grade Ⅱ and one (15%) case of Grade Ⅲ complications. Among them, stoma prolapse was a common complication.

Colostomy for recto-vaginal fistula and rectal stricture is permanent. However, colostomy for patients

unresponsive to medical treatment can be closed when severe proctitis improves sufficiently. Anseline *et al*[19] reported six (43%) colostomy reversals in 14 CRP patients, who were unresponsive to medical treatment. A similar result was observed in the present study. Three (38%) of eight patients with severe bleeding were closed successfully in a mean 9 mo after colostomy. Because the duration of follow-up after fecal diversion was short, many patients who obtained long-term remission of bleeding after colostomy had great potential to reverse the stoma.

In this study, we used a modified SOMA system, which coordinates subjective bleeding symptoms and objective accurate hemoglobin level. According to the system, we suggest that mild to moderate hemorrhagic CRP can be managed by medical or endoscopic treatment. However, for severe refractory bleeding colostomy should be considered to prevent development of serious complications. The scoring system will guide physicians in primary care to evaluate patient condition according to hemoglobin level, and then choose the appropriate treatment. Having a routine and easy protocol can reduce treatment-related delays and avoid unnecessary morbidity[7].

In this study, 44 (94%) CRP patients enrolled had gynecological cancers, so most fistulas were documented in women. In western countries, patients with prostate cancer are the dominant population receiving pelvic radiation, and CRP is mainly reported in prostate cancer[4,12]. However, prostate cancer receives only external beam radiation such as 3D conformal radiotherapy or intensity-modulated radiotherapy, and it does not receive intra-cavity brachytherapy, thus, fewer fistulas are observed. According to our clinical practice, intra-cavity radiation can result in more fistulas and other severe adverse radiation-related symptoms than external beam radiation can.

Although the colostomy group had more severe bleeding than the conservative treatment group, which could have resulted in selection bias, it achieved dramatically better control of bleeding, higher increased hemoglobin level, and improved quality of life compared with the conservative treatment group. These results have shown the advantages of diverting colostomy in treating severe CRP bleeding. Topical formalin or APC was not used in all the patients in the conservative treatment group, because some patients in China have not sufficient knowledge of CRP, poor compliance with physicians' advice, and poor economic status. Thus, they choose to continue self-enemas at home, when recurrent bleeding occurs. In addition, this study was limited by its non-randomized, retrospective design and small sample size. Additional randomized prospective studies of diverting colostomy are needed to confirm our findings.

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COMMENTS

Background

Chronic radiation proctitis (CRP) occurs in 5%-20% of patients receiving radiotherapy for pelvic malignant tumors. Mild to moderate CRP is usually self-limiting and easy to manage, but severe and refractory bleeding is still problematic, especially in cases requiring blood transfusions and that are life threatening. Furthermore, endoscopic treatment can cause severe side effects and only limited efficacy can be obtained for severe CRP. Thus, a simple and safe treatment with fewer complications to save life and relive symptoms is mandatory.

Research frontiers

Diverting colostomy has been reported previously, mainly for severe CRP complications. However, unlike formalin or argon plasma coagulation, colostomy is now not widely used for severe bleeding in CRP patients. The issue of colostomy is not well studied to date. To the best of our knowledge, no study has compared diverting colostomy to conservative measures in treating severe hemorrhagic CRP.

Innovations and breakthroughs

In this series, the authors reported their experience that diverting colostomy was a simple, effective and safe procedure for severe hemorrhagic CRP. Furthermore, they found that colostomy improved quality of life and reduced serious complications secondary to radiotherapy, while conservative medical and endoscopic treatments did not show efficacy in severe CRP patients.

Applications

Diverting colostomy is a simple and safe procedure that can be performed in most medical centers. The authors also developed a modified Subjective Objective Management Analysis system, which coordinates subjective bleeding symptoms and objective accurate hemoglobin level, to guide physicians in primary care to evaluate patient condition according to hemoglobin level, and then choose the appropriate treatment. Having a routine and easy protocol can reduce treatment-related delays and avoid unnecessary morbidity.

Terminology

The underlying causes of CRP are endarteritis obliterans and progressive submucosal fibrosis due to radiotherapy. Diverting colostomy can reduce bacterial contamination and decrease irritation injury by the fecal stream, and can gain time to reduce any radiation reaction to protect injured tissue. Colostomy can also accelerate the course of fibrosis and relieve severe proctitis rapidly, which may prevent deep ulcers progressing to fistulas.

Peer-review

This is a single center, controlled, and retrospective case series of severe CRP patients who received diverting colostomy. Colostomy can relieve most of severe bleeding rapidly and unexpected, colostomy can also reduce serious CRP complications, including remission of long-term perianal pain, transfusion-dependent bleeding and fistula.

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ORIGINAL ARTICLE

Observational Study

Clinical study of anesthetization by dezocine combined with propofol for indolent colonoscopy

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Abstract

AIM: To assess the use of dezocine combined with propofol for the anesthetization of patients undergoing indolent colonoscopy.

METHODS: A cross-sectional survey of patients undergoing indolent colonoscopy in the Xinjiang People's Hospital was conducted from April 1 to April 30, 2015. The survey collected patient general information and anesthesia data, including overall medical experience and pain management. Thirty minutes after colonoscopy surgery, samples of venous blood were collected and the biochemical indicators of gastrointestinal function were analyzed.

RESULTS: There were 98 female and 62 male respondents. Indolent colonoscopy was found to be more suitable for mid to older-aged patients. The necessary conditions for the diagnosis of digestive diseases were required in 65 of the 73 inpatients. Adverse reactions to the intraoperative process included two cases of body movement and two cases of respiratory depression. Gastrin and vasoactive intestinal peptide levels were slightly increased. However, somatostatin and endothelin levels were slightly decreased.

CONCLUSION: This study revealed that dezocine combined with propofol can be successfully used for the anesthetization of indolent colonoscopy patients without pain and should be widely used.

Key words: Dezocine; Propofol; Colonoscopy; Patient

assessment; Anesthetization; Cross-sectional

Core tip: A cross-sectional survey of patients undergoing indolent colonoscopy was carried out to study the clinical effect of anesthetization by dezocine combined with propofol. Thirty minutes after colonoscopy surgery, samples of venous blood were collected and the biochemical indicators of gastrointestinal function were analyzed. Indolent colonoscopy was found to be more suitable for mid to older-aged patients. Gastrin and vasoactive intestinal peptide levels were slightly increased. However, somatostatin and endothelin levels were slightly decreased. This study revealed that dezocine combined with propofol can be successfully used for the anesthetization of indolent colonoscopy patients without pain and should be widely used.

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INTRODUCTION

Colonoscopy is an endoscopic examination of the large intestine and the distal part of the small intestine. It provides a diagnosis and therapeutic opportunity for colorectal lesions[1-4]. At present, colonoscopy remains the gold standard investigation for inspecting colorectal lesions such as adenomas, cancer or inflammation. In many situations, it remains preferable to other imaging examinations such as computed tomography (CT) colonography or barium enema due to the capacity to intervene and sample or remove the pathology encountered.

Unlike sigmoidoscopy which can get to the distal portion (about 600 mm) of the colon, colonoscopy provides an investigation of the full colon (1200-1500 mm)[5,6]. The pain is not caused by the scope of the insertion but rather by the inflation of the colon to allow the inspection. The scope itself is essentially a long, flexible tube about a centimeter in diameter, *i.e.*, around the diameter of the little finger, which is less than the diameter of an average stool[7-9].

Increasingly, doctors like to operate on totally anesthetized patients because it allows for a calmer examination without perceived pain or discomfort. Compared with general anesthesia, twilight sedation is safer and it also allows the patient to follow simple commands and even to watch the operation on a monitor. Therefore, twilight sedation is generally best and the operator should not rush, even although the examination may cause discomfort. Tens of millions of people need colonoscopies annually and yet many of them refuse because of concerns about the procedure[9]. Propofol is a short-acting, intravenously administered hypnotic/amnestic agent. It is usually used in the induction and maintenance of general anesthesia, sedation for mechanically ventilated adults and procedural sedation. Propofol is also used in veterinary medicine[9-11].

Recently, propofol has replaced sodium thiopental for the induction of anesthesia in many hospitals because anabiosis is faster and "clearer" using propofol. Propofol is not a pain medication so it may be combined with opioids such as fentanyl to alleviate pain. Whether this is always necessary is unclear.

Propofol has been known as "milk of amnesia" (a play on milk of magnesia) because the intravenous preparation has a milk-like appearance. It is on the World Health Organization Model List of Essential Medicines. More than 50 countries have approved it for use and generic versions are available[11-15]. Some patients may develop propofol infusion syndrome (PRIS), a rare syndrome that affects patients who undergo long-term treatment with high doses of propofol (> 4 mg/kg per hour for more than 24 h). It can result in cardiac failure, kidney failure, metabolic acidosis and rhabdomyolysis and is often fatal. High blood potassium, high blood triglycerides and liver enlargement, possibly caused by either direct mitochondrial respiratory chain inhibition or impaired mitochondrial fatty acid metabolism, are also key features. Children are more likely to develop PRIS and critically ill patients who receive glucocorticoids and catecholamines are at high risk. The main treatment is supportive therapy[9,16,17]. Early recognition of the syndrome and discontinuation of the propofol infusion reduces morbidity and mortality.

Dezocine is an opioid analgesic which was first synthesized in 1970. It is a mixed agonist/antagonist of opioid receptors and has the effects of analgesic action and euphoria. However, it is a silent antagonist of the κ-opioid receptor and thus has no side effects such as hallucinations or dysphoria[18]. Dezocine was patented by the American Home Products Corp. in 1978. Clinical trials ran from 1979 to 1985 before its approval by the United States Food and Drug Administration in 1986. The use of dezocine was discontinued in the United States in 2011 but it is commonly used after surgery in China.

This study analyzed the clinical manifestations after the administration of dezocine combined with propofol in the anesthetization of indolent colonoscopy patients to assess its effects on gastrointestinal function. The incidence of adverse reactions and improvement of patient tolerance were also studied.

A cross-sectional survey was used to collect epidemiological evidence. The use of regularly collected data provides large cross-sectional studies at little or no expense[19-21], which is an important advantage

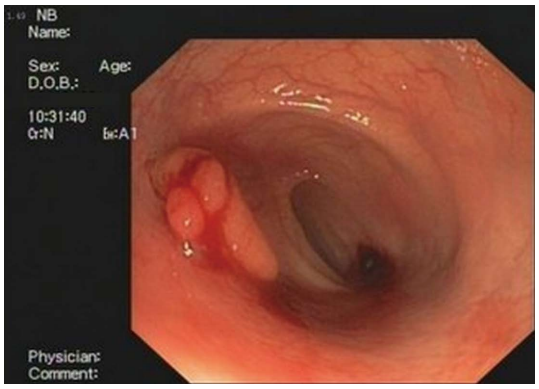

Figure 1 Colonoscopy image.

over other styles of epidemiological studies. A natural progression has been suggested from cheap cross-sectional studies of regularly collected data that can put forward hypotheses. However case-control studies test more specific hypotheses, cohort studies and trials cost much more and take much longer but provide stronger evidence. A cross-sectional survey is conducted to examine a specific group to determine whether alcohol consumption is correlated with cirrhosis being investigated. If alcohol is related to cirrhosis, this would support the hypothesis that alcohol may induce cirrhosis.

MATERIALS AND METHODS

General information

After obtaining institutional review board approval and informed consent from the participants or their guardians, 160 consecutive eligible patients previously diagnosed with indolent colonoscopy were enrolled in the study. All the patients had an ASA physical status of Ⅰ or Ⅱ and were aged between 1 and 68 years old.

All the experiments were performed under the supervision of the Patients' Experimental Ethics Committee of the Beijing Army General Hospital (Beijing, China). The cross-sectional survey, conducted from April 1 to April 30, 2015, recruited patients undergoing indolent colonoscopy in the Xinjiang People's Hospital. Patients with severe organ damage and drug allergies were excluded. This department does not operate on patients aged over 70 or under 1 year, or with a body mass index over 28 or under 18.

General epidemiological data and a history of neurological anesthetic drugs, including intravenous injection or oral drugs, were recorded.

Anesthesia methods

After identification of the patients, intravenous access to the appropriate area was established. All patients received 5 mg of anisodamine, avoiding microvascular spasm. Baseline hemodynamic data were recorded on arrival in the operating room after placement of routine monitors.

Anesthesia was induced with an intravenous injection of 5 mg of dezocine. Ten minutes later, 1.5-2.0 mg/kg of propofol was administered intravenously to induce anesthesia. The dose of propofol was adjusted to maintain the heart rate and blood pressure within 20% of the pre-induction values. No opioids were given intraoperatively. All patients received standardized colonoscopy by a practicing physician.

Evaluation index

The intraoperative index included the time the patient took to lose consciousness (from the induction of anesthesia until the eyelash conditioned reflex disappeared) and the total dosage of propofol.

The postoperative index included the length of time from the end of the colonoscopy surgery to the patient waking up and the patient's pain score in the observation room 30 min after surgery. Adverse reactions were investigated during two phases: the incidence of body movements and respiratory depression in the intraoperative phase and the incidence of vomiting and dizziness or headache in the postoperative phase.

Laboratory testing

Samples of venous blood were collected thirty minutes after the colonoscopy surgery. The biochemical indicators gastrin, vasoactive intestinal peptide, somatostatin and endothelin were tested using the radioimmunoassay (RIA) method. The RIA kits were purchased from the Chinese People's Liberation Army General Hospital, RIA Technology Development Center Institute (East Immunity Institute of Technology).

Statistical analysis

All data are represented as the means x ± SD (x ± s) of three or more independent experiments. Data with a positively skewed distribution were logarithmically transformed into a normal distribution. If the data were homogenous, analysis of variance, the Student-Newman-Keuls test and Pearson's correlation were used. If the data were not homogenous, the Kruskal-Wallis test, Games-Howell test and Spearman's correlation analysis were used. All the analyses were carried out using SPSS17.0 software (SPSS Inc., Chicago, IL, United States). Values below 0.05 were considered to be statistically significant.

RESULTS

Population investigation

From April 1 to April 30, 2015, 160 patients who had undergone dezocine combined with propofol in the anesthetization of indolent colonoscopy took part in the cross-sectional survey. Table 1 summarizes the patients' characteristics. There were 98 females and 62 males; therefore, there were more than 1.6 times

Table 1 Sample characteristics of patients receiving dezocine combined with propofol

Items	Value
Gender	
Male	62
Female	98
Age (yr)	
Median age	43
Maximum age	68
Minimum age	1.3
Average age	48
Modal age	52
Source	
Outpatients	87
Inpatients	73
Feedback from all patients	
Good medical experience	92
Normal medical experience	58
Bad medical experience	9

Table 2 Management survey for operations conducted in this study

	Value
Anesthesiologists' (ASA) Grading	
P 1	89
P 2	62
P 3	9
P 4, 5 or 6	0
Adverse reactions	
Body movement	2
Respiratory depression	2
Vomiting	2
Dizziness	3
Exhaustion and fatigue	12
Pain management	
Level 0-3	132
Level 4-6	28
Above level 7-10	0

as many females as males. They ranged from 1 to 68 years of age, with a median of 43, an average of 48 and a mode of 52 years. These figures indicate that indolent colonoscopy was more common among mid to older-aged patients.

There were 87 outpatients and 73 inpatients. For the purpose of health screening, more people chose indolent colonoscopy, especially the older patients. The necessary conditions for a diagnosis of digestive disease were required in 65 of the 73 inpatients (89.04%).

The patients' feedback about their overall medical experience indicated that 92 had a good medical experience, 58 had an average experience, but 9 had a bad experience and 1 had a very bad experience, which was concentrated in the younger age group. Only one child had no statistical significance.

Management survey for operation

According to the American Society of Anesthesiologists (ASA) grading, 89 patients were classified as P1, 62 as P2, and 9 as P3, but no patient was classified as P4, 5 or 6. All diagnoses met the standard clinical symptoms of the ASA.

The ASA physical status class risk stratification system is dependent on comorbid conditions that are a threat to life or limit activity, thus helping to predicting preoperative risks. They are as follows: (1) P1 - normal healthy patient; (2) P2 - patient with mild systemic disease; (3) P3 - patient with severe systemic disease; (4) P4 - patient with severe systemic disease that is a constant threat to life; (5) P5 - moribund patient who is not expected to survive without the operation; and (6) P6 - declared brain-dead patient whose organs are being removed for donor purposes. All the patients were assessed by the anesthetist before surgery and not by self-reporting.

Among the 160 anesthetized patients, two episodes of postoperative emesis were recorded which occurred after propofol anesthesia. No patient required additional medication for pain in the post-anesthesia care or normal unit. The anesthesia experience perceived by the parents of the children was more appropriate for dezocine combined with propofol. However, three patients reported dizziness 30 min after the operation.

Intraoperative adverse reactions included two cases of body movement and two cases of respiratory depression. The assessment and pain management methods were based on the World Health Organization classification. One hundred and thirty-two patients reported a pain level under 3. No patients reported a higher level of pain; however, 12 patients reported feeling exhausted and fatigued. These results are shown in Table 2.

Length of operation

The mean duration of surgery was 52 ± 24 min, the induction time was 10 ± 4 min and the duration of maintenance was 60 ± 23 min.

Laboratory results

Thirty minutes after the colonoscopy surgery, samples of venous blood were collected and four meaningful biochemical indicators of gastrointestinal function were assessed, as shown in Table 3. The results showed that gastrin and vasoactive intestinal peptide levels were slightly increased, whereas somatostatin and endothelin levels were slightly decreased.

Gastrin can increase gastric mucosal blood flow and protect the gastric mucosa. Vasoactive intestinal peptide can cause the relaxation of the pyloric sphincter. The decrease in somatostatin and endothelin levels helped to inhibit the relaxation of the smooth gastric muscles.

DISCUSSION

General anesthesia refers to a medically induced coma

Table 3 Biochemical indicators of gastrointestinal function

	Detection value	Reference value	Comparison
Gastrin (μg/mL)	88.94 ± 18.77	63.12 ± 28.71	Up
Vasoactive intestinal peptide (pg/mL)	58.33 ± 4.22	35.25 ± 3.12	Up
Somatostatin (pg/mL)	79.42 ± 4.26	108.25 ± 5.12	Down
Endothelin (pg/mL)	36.77 ± 9.12	48.91 ± 10.1	Down

with the lack of protective reflexes caused by one or more general anesthetic agents[1,12,21]. Various kinds of medications may be administered, with the aim of amnesia, analgesia, ensuring unconsciousness, relaxing the skeletal muscles and loss of control of the autonomic nervous system reflexes[9]. The optimal combination of these agents for any given patient and operation is usually chosen by the anesthesiologist or another provider, such as an anesthesiology assistant or nurse anesthetist, in consultation with the patient and the doctor conducting the operation.

Colonoscopy is the standard procedure for the diagnosis, screening, treatment and follow-up of many colorectal diseases[22,23]. Although some patients can tolerate the colonoscopy procedure without sedation or analgesics, the use of drugs in some patients is associated with stress. There are difficulties in determining an optimal dose for sedation and monitoring patients adequately during the procedure[24-27]. Many patients require intravenous benzodiazepines and opiates, which are associated with amnesic, anxiolytic and sedative properties. Combined administration of benzodiazepines and opioids has several undesirable effects, including a delay of several minutes from the time of injection before the drugs exert their effects, amnesia and the risk of respiratory depression[7,28,29].

Hospitals increasingly prefer to use fospropofol for colonoscopy. Fospropofol (trade name Lusedra) is an intravenous sedative-hypnotic agent. It is currently used for the sedation of adult patients undergoing diagnostic or therapeutic operations such as endoscopy. Some water-soluble prodrugs of propofol have been developed, of which fospropofol is known as the most fit for clinical development to date. Fospropofol is often administered combined with an opioid such as fentanyl. As a prodrug of propofol, it is metabolized into propofol by alkaline phosphatases. Theoretically, one millimole (mmol) of propofol may be generated for each mmol of fospropofol sodium administered; 1.86 mg of fospropofol sodium is the molar equivalent of 1 mg propofol[30-33].

Dezocine can be administered intravenously or intramuscularly. Dezocine is an effective painkiller compared with meperidine (pethidine) and a more effective analgesic than pentazocine but may cause greater respiratory depression. It is an effective drug for the treatment of pain but side effects such as dizziness restrict its clinical use and it also causes opioid withdrawal syndrome in patients already using other opioids[34].

A study conducted by the Department of Anesthesia, Xishan People's Hospital of Wuxi City in Jiangsu province explored the clinical effect of dezocine combined with propofol in the anesthetization of 60 patients undergoing indolent enteroscopy between July 2012 and June 2014. The patients were randomly divided into a research group that received dezocine combined with propofol and a control group that received fentanyl combined with propofol[33-36]. The total dosage of propofol in the research group was less than that in the control group ($P < 0.05$), the awakening time in the research group was shorter than in the control group ($P < 0.05$) and the number of adverse effects during and after surgery was lower in the research group than in the control group ($P < 0.05$). Dezocine combined with propofol applied in the anesthetization of indolent enteroscopy can have a remarkable effect, improve operational safety and decrease the occurrence of adverse reactions. Our results were consistent with those of this study.

According to the results of a study by the Department of Anesthesiology at the First Hospital of Quanzhou, age was an important influence on the pharmacodynamics of propofol in patients receiving propofol combined with dezocine while undergoing colonoscopy[37]. However, the results from the Department of Gastroenterology at the Second People's Hospital of Fujian province showed that endoscopic mucosal resection under painless colonoscopy shortened the recovery time, increased the success rate and improved the satisfaction rate of older patients who received propofol and fentanyl. Endoscopic mucosal resection under painless colonoscopy is an easy, safe and effective therapy for colorectal polyps.

Thirty minutes after colonoscopy surgery, samples of venous blood were collected and the biochemical indicators gastrin, vasoactive intestinal peptide, somatostatin and endothelin were tested. There was an increase in the plasma contents of gastrin and vasoactive intestinal peptide ($P < 0.05$) and a decrease in somatostatin and endothelin ($P < 0.05$) compared with the standard reference values[38-40].

Anesthesia with dezocine combined with propofol evidently increases gastric mucosal blood flow, suggesting that it has a regulative effect on gastrointestinal function. The use of dezocine combined with propofol might change plasma brain-gut peptides levels and may be useful.

COMMENTS

Background

Increasingly, doctors like to operate on totally anesthetized patients because it allows for a calmer examination without perceived pain or discomfort. Twilight sedation is safer than general anesthesia and allows the patient to follow simple commands and even to watch the operation on a monitor. Therefore,

twilight sedation is generally best and the operator should not rush, even although the examination may cause discomfort. Tens of millions of adults need colonoscopies annually and yet many refuse because of concerns about the procedure.

Research frontiers

This study assessed the use of dezocine combined with propofol for the anesthetization of patients undergoing indolent colonoscopy for the assessment of gastrointestinal function. A cross-sectional survey of patients undergoing indolent colonoscopy in the Xinjiang People's Hospital was conducted from April 1 to April 30, 2015. The survey collected general information and anesthesia data, including overall medical experience and pain management.

Innovations and breakthroughs

Indolent colonoscopy was found to be more suitable for mid to older-aged patients. Somatostatin and endothelin levels were slightly decreased and patients had a good medical experience.

Applications

Gastrin and vasoactive intestinal peptide levels were slightly increased. However, somatostatin and endothelin levels were slightly decreased. This clinical study of dezocine combined with propofol indicates that it is a successful method for the anesthetization of indolent colonoscopy patients without pain and should therefore be widely used.

Peer-review

In this study, the authors assessed the use of dezocine combined with propofol for the anesthetization of patients undergoing indolent colonoscopy for the assessment of gastrointestinal function. The study collected general information and anesthesia data, including overall medical experience and pain management. This clinical study of dezocine combined with propofol indicates that it is a successful method for the anesthetization of indolent colonoscopy patients without pain and should therefore be widely used.

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CASE REPORT

Successful treatment of ileal ulcers caused by immunosuppressants in two organ transplant recipients

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Author contributions: Guo YW and Gu HY contributed equally to this work; Guo YW, Gu HY, Abassa KK, Lin XY, and Wei XQ analyzed the data and diagnosed and treated the patient; Guo YW, Gu HY, Abassa KK and Wei XQ wrote the paper.

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Abstract

Although gastroduodenal ulcers are common in solid organ transplant patients, there are few reports on multiple giant ulcers in the distal ileum and ileocecal valve caused by immunosuppressants Herein, we report on a liver transplant recipient and a renal transplant recipient with multiple large ulcers in the distal ileum and ileocecal valve who rapidly achieved ulcer healing upon withdrawal of sirolimus or tacrolimus and administration of thalidomide. In case 1, a 56-year-old man with primary hepatocellular carcinoma had received a liver transplantation. Tacrolimus combined with sirolimus and prednisolone was used as the anti-rejection regimen. Colonoscopy was performed because of severe abdominal pain and diarrhea at post-operative month 10. Multiple giant ulcers were found at the ileocecal valve and distal ileum. The ulcers healed rapidly with withdrawal of sirolimus and treatment with thalidomide. There was no recurrence during 2 years of follow-up. In case 2, a 34-year-old man with end-stage kidney disease received kidney transplantation and was put on tacrolimus combined with mycophenolate mofetil and prednisolone as the anti-rejection regimen. Twelve weeks after the operation, the patient presented with hematochezia and severe anemia. Colonoscopy revealed multiple large ulcers in the ileocecal valve and distal ileum, with massive accumulation of fresh blood. The bleeding ceased after treatment with intravenous somatostatin and oral thalidomide. Tacrolimus was withdrawn at the same time. Colonoscopy at week 4 of follow-up revealed remarkable healing of the ulcers, and there was no recurrence of bleeding during 1 year of follow-up. No lymphoma, tuberculosis, or infection of cytomegalovirus, Epstein-Barr virus, or fungus was

found in either patient. In post-transplantation cases with ulcers in the distal ileum and ileocecal valve, sirolimus or tacrolimus should be considered a possible risk factor, and withdrawing them or switching to another immunosuppressant might be effective to treat these ulcers.

Key words: Ileal ulcers; Liver transplantation; Kidney transplantation; Sirolimus; Tacrolimus

Core tip: There are few reports available on ileal ulcers caused by immunosuppressants. Herein, we report a liver transplant recipient and a renal transplant recipient who had multiple large ulcers in the distal ileum and ileocecal valve. Ulcers rapidly healed after withdrawal of sirolimus or tacrolimus and administration of thalidomide. No lymphoma, tuberculosis, or infection of cytomegalovirus, Epstein-Barr virus, or fungus was found in either patient. There was no recurrence of ulcers or organ rejection. In some post-transplantation cases with ileal ulcers, sirolimus or tacrolimus should be considered as a risk factor because of their inhibitory effects on wound healing. Withdrawing them or switching to other immunosuppressants might be effective.

Guo YW, Gu HY, Abassa KK, Lin XY, Wei XQ. Successful treatment of ileal ulcers caused by immunosuppressants in two organ transplant recipients. *World J Gastroenterol* 2016; 22(24): 5616-5622 Available from: URL: http://www.wjgnet.com/1007-9327/full/v22/i24/5616.htm DOI: http://dx.doi.org/10.3748/wjg.v22.i24.5616

INTRODUCTION

Solid organ transplant recipients are susceptible to a variety of gastrointestinal (GI) complications, one of which is ulcer disease[1-5]. Most of these ulcers are located at the anastomotic stoma or gastroduodenum; ulcers at the intestine or colon are rare. In addition to *Helicobacter pylori* infection and ischemia, infection with cytomegalovirus (CMV), Epstein-Barr virus (EBV), mycobacteria, and fungus can contribute to ulcer disease in transplant recipients[1-5]. A few reports suggest that the use of immunosuppressants, especially high-dose immunosuppression after transplantation, might correlate with impairment of the gastrointestinal tract[6,7]. Herein, we report a liver transplant recipient and a renal transplant recipient with multiple large ulcers in the distal ileum and ileocecal valve who rapidly achieved ulcers healing upon withdrawal of sirolimus or tacrolimus and administration of thalidomide.

CASE REPORT

Case 1

A 56-year-old man with primary hepatocellular carcinoma received orthotopic liver transplantation in our hospital. Prior to the transplantation, he was diagnosed with chronic hepatitis B virus (HBV), Child-C liver function, and elevated blood glucose. No history of renal or cardiac disease or mycobacterium tuberculosis (TB) infection was noted. He denied smoking and drinking. Tacrolimus combined with sirolimus and prednisolone was used as the anti-rejection regimen. The dose of tacrolimus and sirolimus was adjusted according the drug serum concentration. The patient recovered and liver function improved to normal level. Entecavir was prescribed to prevent HBV reinfection.

After administration of the immunosuppressants, the patient began to develop mild peri-umbilical pain and diarrhea, which were tolerable at the time. Probiotics and antispasmodic treatment seemed not so effective. At 10 mo post-operation, he was admitted to our hospital because of severe diarrhea and abdominal pain. There were 7-10 bowel movements per day, with mucous blood stool. On physical examination, blood pressure, heart rate, and temperature were normal. There was slight tenderness on the peri-umbilicus area without rebound tenderness. Complete blood count revealed a leukocyte count of 11.50 × 10^9/L, erythrocyte count of 4.50 × 10^{12}/L, hemoglobin level of 121 g/L, platelet count of 295 × 10^9/L, neutrophils of 76.4%, and lymphocytes of 16.3%. Leukocytes and erythrocytes were found in the stool. The ratio of coccus to bacillus in stool was 1:9. Serum glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase, total bilirubin, BUN, creatine, and electrolytes were within normal range. Serum 1-3-β-D dextran was < 10 pg/mL. Blood and stool cultures for fungus and bacteria showed no growth. Chest computed tomography (CT) scan revealed no lesions and serum T-SPOT.TB was negative. Immunoglobulin (Ig)M and IgG of the EB virus were negative. Abdominal enhanced CT scan showed thickening of the distal ileum and ileocecal region, suggesting inflammatory lesions. The spleen was slightly enlarged, muddy stones were found in the common bile duct, and no enlarged lymphonodus was noted. Chronic erosive gastritis with negative *Helicobacter pylori* infection was confirmed through esophagogastroduodenoscopy. Colonoscopy revealed multiple giant and deep ulcers in the ileocecal valve and distal ileum, with polypoid hyperplasia. The length of the largest ulcer was up to 5.0 cm (Figure 1A-C). Histopathology of biopsy specimens revealed benign ulcers and chronic inflammation with non-caseous granulomas (Figure 2A and B), without signs of fungus and parasites infection. The immunohistochemical study was negative for CMV infection. EBV encoded

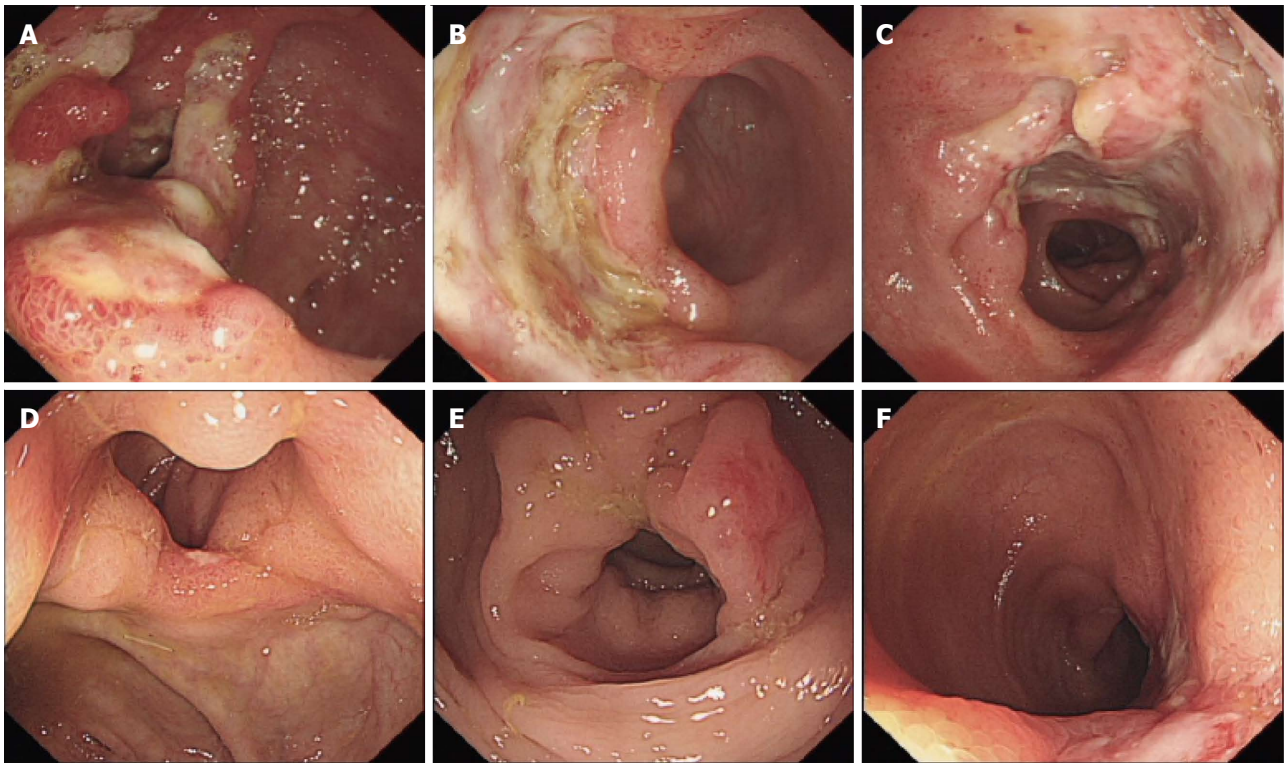

Figure 1 Colonoscopic images of case one. A-C: Multiple giant and deep ulcers in the ileocecal valve and distal ileum, with polypoid hyperplasia; D-F: Rapid healing of the ulcers in the ileocecal valve and distal ileum and only two healing 2 stage ulcers left.

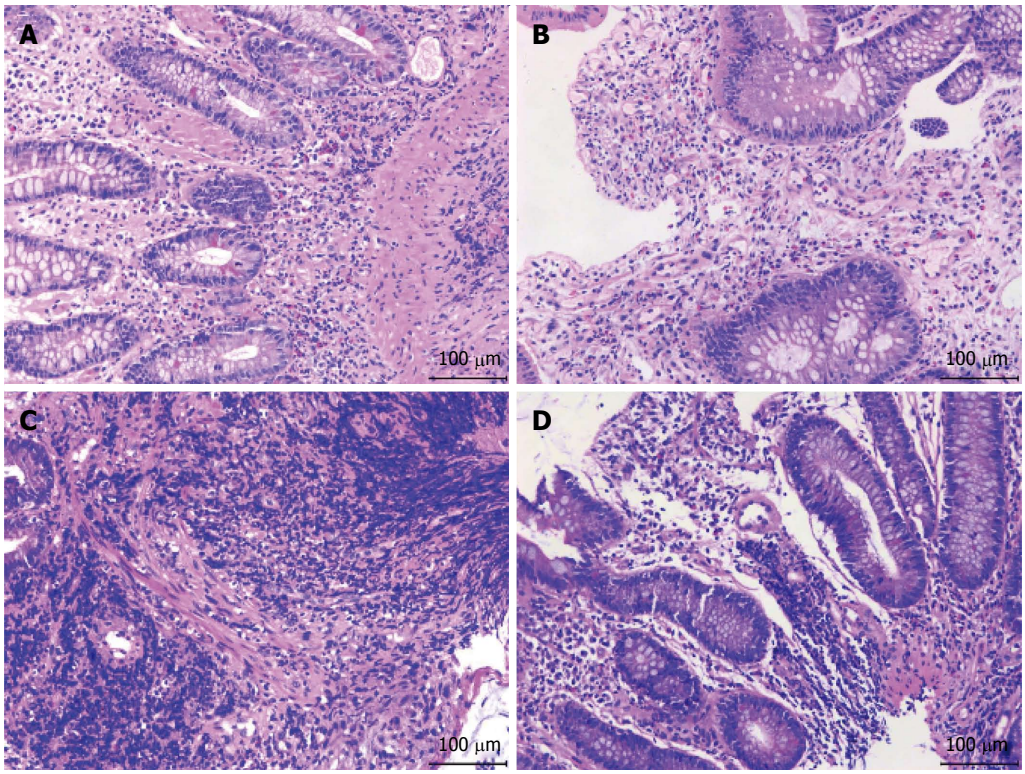

Figure 2 Photomicrograph of biopsy specimens. A and B: Biopsy specimens of ulcers from case one; C and D: Biopsy specimens of ulcers from case two. Hematoxylin-eosin staining, magnification × 200.

early small RNA (EBER) was negative by *in situ* hybridization.

Considering sirolimus to have more gastrointestinal complications than tacrolimus in clinical application,

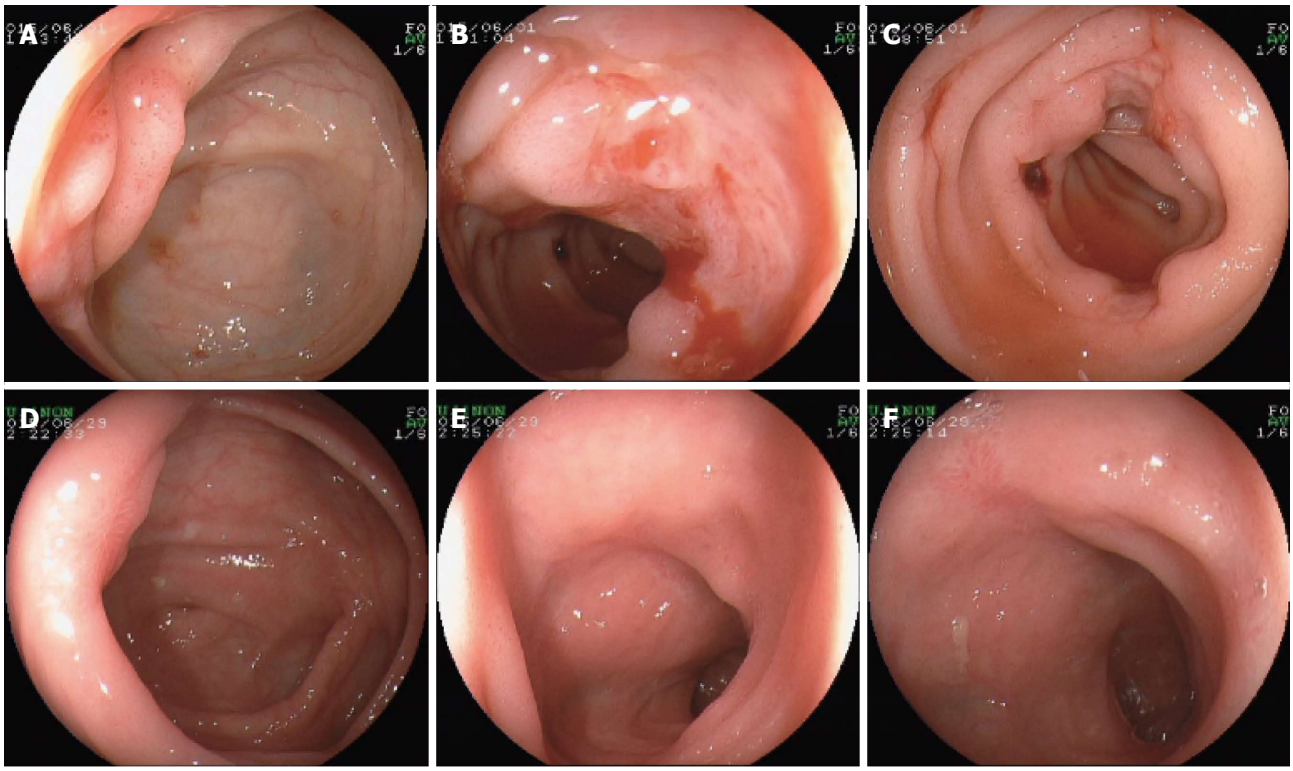

Figure 3 Multiple ulcers in ileocecal valve and distal ileum, with massive fresh blood accumulation (A-C); rapid healing of the ulcers with scar tissue in ileocecal valve and distal ileum (D-F).

sirolimus was withdrawn first. The patient was also put on oral thalidomide at a dose of 100 mg/d for 2 wk and intravenous antibiotics for one week. Diarrhea and abdominal pain were gradually relieved and subsided. Colonoscopy at week 6 of follow-up revealed remarkable healing of the ulcers in the ileocecal valve and distal ileum, and only two healing 2 stage ulcers were found (Figure 1D-F). No organ rejection was noted after withdrawing sirolimus. No recurrence of diarrhea and abdominal pain was noted during the 2 years of follow-up.

Case 2

A 34-year-old man with end-stage kidney disease was admitted to the department of renal transplantation for living-donor kidney transplantation. Except for kidney disease, he had no history of primary liver, heart, or head disease. He denied smoking and drinking. Tacrolimus combined with mycophenolate mofetil and prednisolone was applied as the anti-rejection regimen. The dose of tacrolimus was adjusted according to the drug serum concentration. At the same time, oral ganciclovir, voriconazole, and esomeprazole were used to prevent CMV and fungus infection and esophagogastroduodenal complications. The patient recovered smoothly. Serum creatinine level decreased to 152.0 μmol/L, and urine output was normal.

At postoperative week 12, the patient was admitted to our department because of repeated hematochezia for 2 wk, which was accompanied by dizziness and weakness. No abdominal pain, nausea, or vomiting was observed. On physical examination, blood pressure, heart rate, and temperature were 95/55 mmHg, 102 beats/min, 37.5 ℃, respectively. There was mild tenderness at the peri-umbilicus area and the lower right abdomen, without rebound tenderness. Complete blood count revealed a leukocyte count of 7.45 × 10^9/L, erythrocyte count of 2.05 × 10^{12}/L, hemoglobin level of 57 g/L, and platelet count of 241 × 10^9/L. Mild elevated serum creatinine level of 168.0 μmol/L was noted, with a normal BUN level of 6.8 mmol/L. Prothrombin time, apartprothrombin time, and thrombin time were within the normal range. Laboratory indices about hepatic, cardiac, and respiratory function were all normal. Blood and stool cultures for fungus and bacteria showed no growth. Chest CT scan revealed some pulmonary lesions of previous tuberculosis, and serum T-SPOT.TB was negative. There were no abnormal findings in the abdominal doppler ultrasound. Esophagogastroduodenoscopy was performed first, and only mild gastritis was observed, with negative rapid urease test for *Helicobacter pylori* infection. Colonoscopy revealed multiple ulcers in the ileocecal valve and distal ileum, with massive accumulation of fresh blood. These ulcers were oval and deep, covered with white fur or a blood scab, and the biggest had a diameter of 2.0 cm (Figure 3A-C). Histopathology revealed chronic inflammation with a large number of lymphocytes infiltration (Figure 2C

and D), without signs of fungus or parasite infection. Immunohistochemistry stain for CMV was negative. EBER was also negative by *in situ* hybridization.

The bleeding lessened and eventually ceased after treatment with intravenous somatostatin (1.2 mg/d) and oral thalidomide (100 mg/d) for 5 d. At the same time, because the multiple intestinal ulcers may be due to immunosuppressors, tacrolimus was withdrawn, and cyclosporine combined with mycophenolate mofetil and prednisolone were administered. Additionally, the patient was put on oral thalidomide at a dose of 100 mg/d for 4 wk. Colonoscopy at week 4 of follow-up revealed remarkable healing of the ulcers with scar tissue in the ileocecal valve and distal ileum (Figure 3D-F). There was no recurrent bleeding during the 1 year of follow-up. In addition, no organ rejection was found after withdrawing tacrolimus.

DISCUSSION

It is well known that solid organ transplantation patients are particularly at risk for GI complications. Severe GI complications such as GI bleeding and GI perforation may negatively influence long-term outcome and become deadly. It had been reported that GI bleeding occurred in 2.3%-6.4% patients after liver transplantation[8,9] and GI perforation in 2.9% patients after renal transplantation[7]. Ulcer diseases are an important cause of GI bleeding, and perforation can manifest with symptoms of abdominal pain or diarrhea. Most of these ulcers are located at the anastomotic stoma or gastroduodenum, whereas ulcers at the intestine or colon are rare. In the two cases described here, deep and large ulcers were located in the ileocecal valve and distal ileum with no lesions in the gastroduodenum. In case 1, there was a longer period of diarrhea and abdominal pain, while case 2 mainly presented with acute and massive GI bleeding.

Differentiation of ulcer diseases in post-transplantation patients is always difficult. Common and uncommon pathogenesis such as *Helicobacter pylori* infection, ischemia, infection of CMV, EBV, mycobacteria, and fungus, and post-transplant lymphoproliferative disorders (PTLDs) should be considered. In the present two cases, ulcers were in the ileocecal valve and distal ileum, and *Helicobacter pylori* infection was ruled out as the pathogen. Clinical manifestation and endoscopic characteristics of ulcers were not consistent with a pathogenesis of ischemia. Because of negative the findings on chest CT scan, serum T-SPOT.TB, and histopathology of ulcers, mycobacteria and fungus infection were also excluded. CMV infection is common in patients with solid organ transplantation, and attention should be paid in cases of gastrointestinal ulcers. In a study of renal transplant patients susceptible to a variety of GI complications, such as infections, ulcer disease, and malignancies, CMV infection occurred in 11% of all patients[1]. Although oral ganciclovir preventive strategy was used in case 2, this treatment might be inefficient in some patients, and atypical symptoms might be present[10,11]. Decreased leukocyte count and interstitial pneumonitis are always found in CMV infection, but in both of our cases, no positive signs about CMV infection were noted in leukocyte count or chest CT scan. More importantly, immunohistochemistry stain for CMV of ulcer biopsy tissue was negative, and both patients recovered without further anti-CMV therapy. Therefore, CMV infection was not considered the cause of ulcers in these two cases.

PTLD was most difficult to be excluded in both patients. This was especially true in case 1, where the ulcers in the ileocecal valve and distal ileum appeared deeper and larger, with surrounding proliferative tissue. PTLD is a severe complication after organ transplantation with a cumulative incidence of 1.1% at 18 mo and 4.7% at 15 years, and it is always associated with EBV infection[12]. Lymph nodes, GI tract, and graft liver are the most common sites of involvement[13]. The involvement of the GI tract could result in deadly perforation and hemorrhage. In both patients, no persistent fever, palpable superficial lymph nodes, enlarged liver, or lymph nodes were found on chest and abdominal CT scan or ultrasound; EBER was negative by *in situ* hybridization. Pathology was also not consistent with the characteristics of PTLD. Furthermore, PTLD usually deteriorates quickly and is difficult to treat. However, the present two patients were stable within 1-2 wk, and the ulcers healed rapidly in 4-6 wk. Taken together, the evidence did not support the diagnosis of PTLD.

Finally, we considered that ulcer development was related to the use of immunosuppressants. Current studies have shown that some immunosuppressants, such as mammalian target of rapamycin inhibitors, had inhibitory effects on wound healing. Sirolimus is the most common drug that can lead to impairment of wound healing, and the most common wound complication is skin or dermal eruption[14-17]. Therefore, the immunosuppressant use was considered to be a possible cause of GI epithelium impairment. Fortunately, both patients recovered quickly after withdrawing sirolimus and tacrolimus, supporting our speculation. In Smith *et al*[6], three liver transplant patients taking sirolimus suffered from gastrointestinal hemorrhage due to complications of gastroduodenal ulcers. The ulcers in two patients healed only after discontinuation of sirolimus, and the third patient died of massive gastrointestinal bleeding[6].

Thalidomide has anti-angiogenic properties and seems effective in some cases of GI bleeding, especially angiodysplasia-related bleeding[18-20]. It is also used in some inflammatory and ulcerative diseases like inflammatory bowel disease and some skin and oral ulcers, because of its anti-inflammatory and immunomodulatory effects[20-22]. In our clinical practice, thalidomide is effective in some unexplained

and refractory multiple ulcers of intestine and related GI bleeding. It also seemed to work in our present two patients. Thalidomide was administrated at a dose of 100 mg/d for 2 wk and 4 wk respectively, and no severe side effects were found.

In summary, some types of immunosuppressants, such as sirolimus and tacrolimus, can lead to impairment of the GI track and sometimes to the development of severe ulcers. Withdrawing them or switching to other immunosuppressants might be effective to treat these ulcers.

COMMENTS

Case characteristics

A 56-year-old man presented with severe diarrhea and abdominal pain after orthotopic liver transplantation, and a 34-year-old man presented with hematochezia and severe anemia after living-donor kidney transplantation.

Clinical diagnosis

Multiple giant ulcers in the distal ileum and ileocecal valve were caused by immunosuppressants.

Differential diagnosis

Common and uncommon pathogenesis of gastrointestinal (GI) ulcers in solid organ transplant recipient, such as *Helicobacter pylori* infection, ischemia, infection of cytomegalovirus (CMV), Epstein-Barr virus (EBV), mycobacteria and fungus, and post-transplant lymphoproliferative disorders (PTLDs), should be considered.

Laboratory diagnosis

Blood and stool cultures for fungus and bacteria showed no growth, and serum T-SPOT.TB was negative.

Imaging diagnosis

Computed tomography (CT)scan revealed no current tuberculosis, and there were no abnormal findings on abdominal enhanced CT or doppler ultrasound.

Endoscopic diagnosis

Colonoscopy revealed multiple giant and deep ulcers in the ileocecal valve and distal ileum.

Pathological diagnosis

Histopathology revealed chronic inflammation without signs of fungus or parasite infection, negative immunohistochemistry stain for CMV, and negative stain for EBV encoded early small RNA by *in situ* hybridization.

Treatment

Sirolimus or tacrolimus was withdrawn, and thalidomide was administrated.

Related reports

Most GI ulcers are located at the anastomotic stoma or gastroduodenum in post-transplant recipients, and ulcers at the intestine or colon are rare. Besides ischemia and infection of CMV, EBV, mycobacteria, and fungus, use of immunosuppressants might contribute to the impairment of gastrointestinal tract.

Term explanation

PTLDs are a severe complication of solid organ and hematopoietic stem cell transplantation, including lymphoproliferative entities varying from reactive hyperplasia to malignant lymphoma. EBV is the main pathogen of PTLD.

Experiences and lessons

In some post-transplantation cases with ileal ulcers, sirolimus or tacrolimus should be considered as a risk factor, and withdrawing them or switching to other immunosuppressants might be effective.

Peer-review

This article is very interesting for those in the field of liver and kidney transplantation. Since ulcers in the distal ileum in solid organ transplant recipients are very rare, experience about the management of these patients is very useful.

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