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REVIEW

Immune therapies in pancreatic ductal adenocarcinoma: Where are we now?

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is one of the deadliest cancers, mostly due to its resistance to treatment. Of these, checkpoint inhibitors (CPI) are inefficient when used as monotherapy, except in the case of a rare subset of tumors harboring microsatellite instability (< 2%). This inefficacy mainly resides in the low immunogenicity and non-inflamed phenotype of PDAC. The abundant stroma generates a hypoxic microenvironment and drives the recruitment of immunosuppressive cells through cancer-associated-fibroblast activation and transforming growth factor β secretion. Several strategies have recently been developed to overcome this immunosuppressive microenvironment. Combination therapies involving CPI aim at increasing tumor immunogenicity and promoting the recruitment and activation of effector T cells. Ongoing studies are therefore exploring the association of CPI with vaccines, oncolytic viruses, MEK inhibitors, cytokine inhibitors, and hypoxia- and stroma-targeting agents. Adoptive T-cell transfer is also under investigation. Moreover, translational studies on tumor tissue and blood, prior to and during treatment may lead to the identification of biomarkers with predictive value for both clinical outcome and response to immunotherapy.

Key words: Drug therapy combination; Immunology; Hypoxia; Checkpoint inhibitor; Inflammation; Pancreatic cancer; Tumor-infiltrating lymphocyte; Transforming growth factor β; Tumor microenvironment

Core tip: Checkpoint inhibitors (CPI) and other immune

therapies remain inefficient when used as single agents in pancreatic ductal adenocarcinoma (PDAC). Here, we present an overview of the biological mechanisms underlying these failures and the lessons learned, giving a rationale for innovative combination therapies. In particular, the latest ongoing studies are attempting to overcome the immunosuppressive microenvironment, the basis of resistance to CPI in PDAC.

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INTRODUCTION

Immunotherapy has paved the way for new therapeutic opportunities in cancer. Cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and programmed cell death-1 (PD-1) are receptors expressed on the surface of T-cells that regulate the duration and the amplitude of immune responses in physiological conditions[1]. CTLA-4 is involved in the priming phase (lymph node) while PD-1 and its ligand PDL-1 are implicated in the effector phase (tumor) (Figure 1). The hijacking of these immunological "checkpoints" by cancer cells is a major mechanism of immune evasion, a better understanding of which led to the clinical development of anti–CTLA-4 and anti–PD-1/PD-L1 mAb with striking efficacy in several malignancies, including chemoresistant tumors. For example, objective responses associated with prolonged survival were observed in 30%-45% of melanomas[2], 15%-20% of lung cancers[3,4], 13% of pre-treated head and neck carcinomas[5], 22%-25% of pre-treated kidney cancers[6], and more than 60% of Hodgkin lymphomas[7] following anti–PD-1/PD-L1 monotherapies, leading to their clinical approval in these indications. However, immunotherapy failed to improve the outcome of patients in some tumor types[8], notably pancreatic ductal adenocarcinoma (PDAC).

Recent epidemiological projections have predicted that PDAC will become the second leading cause of cancer-associated death in the USA and Europe by 2030[9]. PDAC is the gastrointestinal tumor with the poorest prognosis, with 80% of patients having advanced disease at diagnosis and a 5-year survival rate that does not exceed 7%[10]. PDAC is characterized by its resistance to conventional therapies (chemotherapy, targeted therapy and radiotherapy)[11]; thus innovative therapeutic options are crucially needed. Despite hopes raised by the results of immune therapies in other cancers, these strategies have so far been disappointing in PDAC. Nonetheless, an improved understanding of the biology of its microenvironment has recently provided a rationale for innovative therapeutic combinations to unlock PDAC resistance to immune therapy.

The objectives of this review are (1) to present an overview of the immune therapies that have so far been tested in PDAC, (2) to describe the main mechanisms involved in resistance to these therapies, and (3) to introduce the current strategies to overcome this resistance.

FAILURE OF IMMUNE MONOTHERAPIES IN PDAC

Patients with PDAC were treated with anti–PD-1/PD-L1 (pembrolizumab, atezolizumab) and anti–CTLA-4 (ipilimumab) monotherapies in three phase Ⅰ[12–14] and one phase Ⅱ trials[14], respectively. Overall, these studies showed no activity of checkpoint inhibitor (CPI) monotherapies in unselected patients with advanced, pre-treated, progressive PDAC (Table 1).

Nevertheless, PD-1 blockade appears to be efficient in a subset of patients with PDAC harboring a mismatch repair (MMR) deficiency. The MMR machinery is encoded by four key genes (MLH1, MSH2, MSH6, PMS2), which behave as genome safeguards by correcting base mispairs occurring during DNA replication. Loss of MMR results in drastically increased rates of somatic mutations[15,16], potentially translated into neoantigens that can be recognized by the immune system[17,18] rendering them responsive to CPI. MMR deficiency can be caused by inherited germline defect in the case of Lynch syndrome, predisposing to a spectrum of tumors [mainly, colorectal (CRC) and endometrial cancers], or emerge from somatic mutations or promoter methylation (*e.g.,* in *BRAF*-mutated CRC)[19]. Microsatellite instability-high (MSI-H) is the phenotypic evidence of MMR deficiency. Recently, the use of pembrolizumab was approved for MSI-H or MMR-deficient tumors based on five clinical trials[20], which including 149 patients with tumors from 15 primary origins, mostly CRC (91/149). The objective response rate was 39.6%, including complete responses in 7.4%, and 78% of responses lasted more than 6 mo. MSI-H is thus recognized as a predictive biomarker of response to PD-1 blockade[21,22].

Six patients with PDAC were included in a multitumor expansion study of pembrolizumab (12 cancer types) with evidence of clinical benefit (one stable disease, three partial responses, and two complete responses). However, MSI-H is a rare event in PDAC[23] as illustrated by a genetic study on 385 PDAC that reported that hypermutated profiles (all related to MMR deficiency) were found in less than 2% of cases (4 out of 385)[24]. Therefore, the subset of PDAC patients eligible for CPI monotherapy is small.

Beside CPI, other immune therapy strategies (vaccines, oncolytic viruses, TGFβ inhibitors) have been tested and also remained inefficient in PDAC patients when used as monotherapies or in combination with gemcitabine chemotherapy (Table 1). Overall, except

Table 1 Summary of clinical trials of immune therapies (single agent or combination with gemcitabine) in patients with pancreatic ductal adenocarcinoma

Type of immunotherapy	Molecules	Trial	Phase	*n*	Population	Main results
Immune checkpoint inhibitors	PD-L1 (BMS-936559)	Brahmer *et al*[8]	I	14	Advanced PDAC Pre-treated	No objective response
	PD-L1 (atezolizumab)	Herbst *et al*[12]	I	1	Advanced PDAC Pre-treated	No objective response
	PD-1 (pembrolizumab)	Patnaik *et al*[13]	I	1	Advanced PDAC Pre-treated	No objective response
	CTLA-4 (ipilimumab)	Royal *et al*[14]	II	27	Advanced PDAC Pre-treated	No objective response
Therapeutic vaccines	GVAX	Jaffee *et al*[118]	I	14	Resected PDAC Adjuvant Combination with chemoradiotherapy	3 patients remained disease-free for > 25 mo
		Lutz *et al*[119]	II	60	Resected PDAC Adjuvant Combination with chemoradiotherapy	Median disease-free survival: 17.3 mo Median overall survival: 24.8 mo
		Laheru *et al*[120]	II	50	Advanced PDAC Pre-treated Combination with cyclophosphamide	Median overall survival: 4.3 mo
		Lutz *et al*[30]	Pilot Randomized	54	Resected PDAC Neoadjuvant and adjuvant Combination with cyclophosphamide	Arm 1: GVAX alone Arm 2: Cyclophosphamide (intravenous) + GVAX Arm 3: Cyclophosphamide (daily oral) + GVAX Intra-tumoral tertiary lymphoid aggregates PD-1 and PDL-1 upregulation
	CRS 207	Le *et al*[121]	I	7	Advanced PDAC Pre-treated	No objective response
	GVAX + CRS 207	Le *et al*[78]	II Randomized	90	Advanced PDAC Pre-treated	Arm 1: Cyclophosphamide + GVAX + CRS-207 Arm 2: Cyclophosphamide + GVAX No objective response
	Algenpantucel-L	Hardacre *et al*[122]	II	70	Resected PDAC Adjuvant Combination with chemotherapy	Disease-free survival: 62% at 1 yr Overall survival: 86% at 1 yr
	Mutated KRAS peptide	Gjertsen *et al*[123]	I / II	5	Advanced PDAC Pre-treated	No objective response
		Gjertsen *et al*[124]	I / II	48	Advanced PDAC Pre-treated Resected PDAC Adjuvant	No objective response Median overall survival in resected PDAC: 25.6 mo
		Abou-Alfa *et al*[125]	I	24	Resected PDAC Adjuvant	Median disease-free survival: 8.6 mo Median overall survival: 20.3 mo
	Telomerase peptide (GV1001)	Middleton *et al*[126]	III Randomized	1062	Advanced PDAC First line Combination with chemotherapy	Arm 1: chemotherapy alone Arm 2: sequential chemo-immunotherapy Arm 3: concurrent chemo-immunotherapy No benefit on overall survival of adding vaccination to chemotherapy
Oncolytic viruses	Mutated adenovirus (ONYX-15)	Hecht *et al*[127]	I / II	21	Advanced PDAC Pre-treated and first line Combination with chemotherapy	Two partial responses
		Mulvihill *et al*[128]	I	23	Advanced PDAC Pre-treated and first line	No objective response
Anti-transforming growth factor β (TGFβ)	Anti-TGFβ2 (trabedersen)	Oettle *et al*[129]	I / II	37	Advanced PDAC Pre-treated	One complete response

	TGFβ receptor inhibitor (galunisertib)	Melisi *et al*[130]	II Randomized	156	Advanced PDAC Pre-treated and first line Combination with chemotherapy	Arm 1: galunisertib + gemcitabine Arm 2: gemcitabine +placebo No benefit on overall survival of adding galunisertib to chemotherapy

CTLA-4: Cytotoxic T lymphocyte-associated protein 4; PD-1: Programmed cell death-1; PD-L1: Programmed death-ligand 1.

for MSI-H tumors, PDAC are considered to be resistant to single-agent immune therapy.

Reasons why checkpoint inhibitor monotherapies failed to show any activity in pancreatic ductal adenocarcinoma

The "cancer-immunity cycle" theory defines three conditions that are required to obtain an effective anti-tumoral immune response[25]: tumor immunogenicity, T cell recruitment and activation.

Tumor immunogenicity: Immunogenicity is related to the degree of epitope structural difference between tumor and normal cells. The more different the epitope, the more likely to be recognized by T cells[26]. Hence, tumor-associated antigens (TAA) loosely fall into two classes based on their tumoral specificity and immunogenicity: (1) Low (differentiation antigens, overexpressed self-antigens) and (2) high (viral antigens, cancer-germline genes, and neoantigens) tumoral specificity. Neoantigens are peptides generated from non-silent coding mutations in the cancer cell genome and are highly immunogenic. Several studies have shown that tumor mutation load is linked to neoantigen burden and positively correlated with response to immunotherapy[27,28]. Pancreatic cancer has a low mutation load compared to other solid tumors, with an average mutation rate of 1 mutation per megabase (Mb) (compared to 11 mutations per Mb for melanoma), only occasionally yielding neoantigens[29]. Nevertheless, PDAC has an immunogenic capacity as reflected by the presence of T-cell infiltrates and tertiary lymphoid structures in resected PDAC samples[30-32]. Some studies suggest that although the rate of mutations is low, it is sufficient to create highly immunogenic neoantigens, notably through *KRAS* codon 12 mutations[33,34].

Importantly, DNA mutations do not necessarily translate into immunogenicity because both antigen presentation by major histocompatibility complex (MHC) and recognition by the T cell receptor (TCR) with a high affinity are required to induce T cell response, leading to the concept of neoantigen quality. It has been shown that the *fitness* of a neoantigen, *i.e.*, its distance from the wild type sequence coupled with its binding affinity to the TCR, is correlated with the activation of T cells[35]. High-quality neoantigens (mutation-associated or microbial-like sequences) have been associated with longer survival in PDAC, highlighting the fact that the neoantigen quality outweighs the neoantigen quantity in clinical significance[36].

Determining MHC-antigenic structures (*e.g.,* using mass spectrometry) is useful to (1) predict which neoantigen will be recognized by T cells and (2) identify actionable targets to trigger the immune response (*e.g.,* for vaccine strategies)[37-39]. Nonetheless, such approaches are currently limited by the poor performance of neoepitope predictive algorithms. Indeed, less than 5% of predicted neoepitopes actually give rise to a biological response[34]. The Tumor Neoantigen Selection Alliance initiative is a global bioinformatics collaborative effort aiming to develop a software that can best predict immunogenic mutation-associated cancer antigens from patients' tumor DNA[40].

T cells recruitment and activity: The release of tumor neoantigens following cell death[41] allows antigen-presenting cells (APC), such as dendritic cells to uptake and present them to T cells leading to the activation of the latter[42-44]. Secondly, T cells must be recruited into the tumor after trafficking in blood vessels[45] and passing through the endothelial wall[46]. Finally, tumor-infiltrating lymphocytes (TIL) recognize and kill tumor cells[43].

Depending on the histological pattern of TIL, tumors are classified into T-cell inflamed (also known as "hot" tumors) *vs* non-inflamed ("cold") tumors, in which T cells are excluded or absent[47]. Preclinical and clinical evidence suggest that only patients who have T-cell inflamed tumors respond to CPI monotherapy[47]. Most PDAC are thought to belong to the non-inflamed tumor group, displaying low levels of TIL along with low PD-L1 expression, which can account for the poor efficacy of single-agent immune therapies[48-50].

PDAC display an abundant desmoplastic stroma, the extent of which is often greater than the epithelial component of the tumor[51,52]. The stroma is a complex structure composed of extracellular matrix proteins and various cell types including cancer associated fibroblasts (CAF), endothelial cells, and immune cells[52]. This fibrotic barrier was believed to physically impede T cell infiltration[53]. However, recent work using multiplex imaging for spatial analysis of desmoplastic elements in PDAC revealed that collagen I deposits are inversely correlated with TIL numbers[54]. This observation has led to the hypothesis that the stroma may be a chemical rather than a physical barrier[55] (Figure 2). Indeed, PDAC is characterized by a high density of immunosuppressive cells including T regulatory cells (TREG) and myeloid cells [*e.g.* dendritic cells, myeloid derived suppressive cells (MDSC) and M2 macrophages], which are negative prognostic factors[56]. Myeloid cells release TGFβ[57], nitric oxide synthase and arginase, preventing TIL recruitment and activity[56,58]. Tumor hypoxia is a predominant driver in the recruitment of these immune cells through CAF

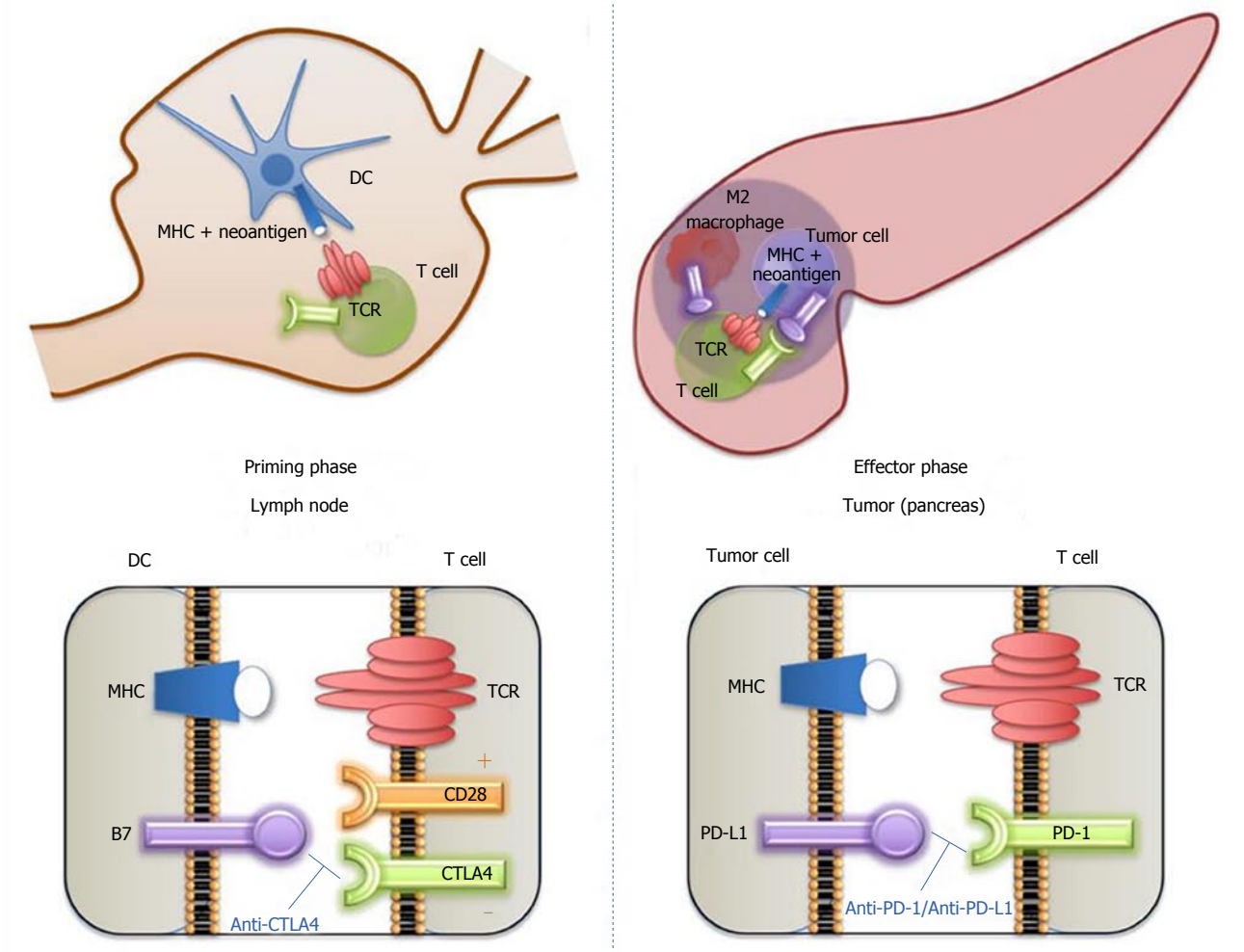

Figure 1 Cytotoxic T lymphocyte-associated protein 4 and programmed cell death-1 biological functions and therapeutic targeting. Cells of the immune system express several surface molecules that are important for immune surveillance and regulation of the immune response. T cell receptor (TCR) is expressed by T cells; it is an antigen-specific molecule that is unique to each T cell clone. Major human compatibility (MHC) molecule is expressed by antigen-presenting cells (*e.g.*, dendritic cell) and display a potential tumor antigen for recognition by the specific TCR. Left panel: When an antigen presented in the context of MHC is recognized by the TCR, interaction of CD28 (expressed by T cell) with B7 (CD80/CD86) molecules provide a co-stimulatory signal leading to T-cell activation. However, depending on the conditions and microenvironment, these T cells can also express various levels of cytotoxic T lymphocyte-associated protein 4 (CTLA-4), a regulatory receptor (immune checkpoint) with a higher binding affinity for B7 than CD28. Therefore, when CTLA-4 is available at the cell surface, it successfully competes for binding with B7, removing the co-stimulatory signal and leading to T-cell downregulation. Tumor cells can then escape the T cell cytotoxic effect (immune evasion). CTLA-4 blockade affects the immune priming phase occurring in the lymph node, by supporting the activation and proliferation of a higher number of effector T cells, regardless of TCR specificity, and by reducing Treg-mediated suppression of T-cell responses. Right panel: T cells also express PD-1 receptor, which has the potential to induce a programmed-death cascade in T cells that mistakenly react to host cells and thereby maintaining self-tolerance. PD-1 ligand, PD-L1, is used by tumor cells to engage the PD-1 receptor and switch off the reaction, inducing immune tolerance to the MHC-presented antigen. PD-L1 can also be expressed by stromal cells (*e.g.*, M2 macrophages). PD-1 blockade works during the effector phase in peripheral tissues (tumor) to restore the immune function of "exhausted" T cells that have been turned off following extended or high levels of antigen exposure. CTLA-4: Cytotoxic T lymphocyte-associated protein 4; DC: Dendritic cell; MHC: Major human compatibility; PD-1: Programmed cell death-1; PD-L1: Programmed death-ligand 1; TCR: T cell receptor.

activation[59-61]. Activated CAF then secrete immunosuppressive cytokines[62,63], such as CXCL12 and IL-6, which promote MDSC recruitment and inhibit effector T cell recruitment.

In addition, although T cell infiltration seems to be necessary for the response to immune therapy, the presence of TIL is not sufficient to induce an effective anti-tumor response[64]. Indeed, TIL activation is required. However, in PDAC, even in the presence of tumor-specific neoepitopes, T cells display a reduced activation signature[34] and most of them are PD-1–positive[65], suggesting that T cell activation is actively suppressed. Notably, not only MDSC but also TREG and CD8-positive γδT cells restrain activation of αβT cells that are directed against the tumor[66]. These deleterious TIL represent approximately 40% of CD8-positive TIL populations in PDAC and may mislead the interpretation of the biological significance of TIL in PDAC. This may enlighten some negative results showing no prognostic impact of T cell infiltration in PDAC[56,64].

Overall, given its low mutational load, low lymphocyte count, the presence of inflammatory cytokines and hypoxia, PDAC displays a unique microenvironment that is unfavorable to immune therapy according to the cancer immunogram and requires combination strategies[67].

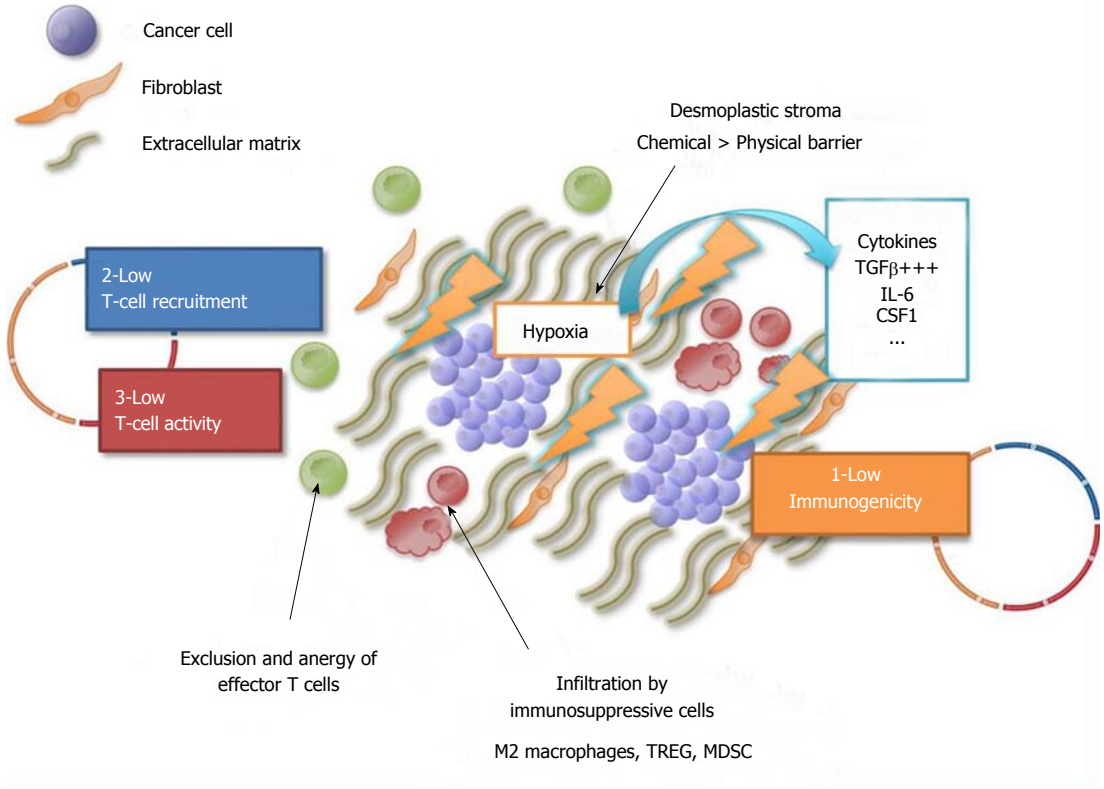

Figure 2 Summary of the mechanisms responsible for pancreatic ductal adenocarcinoma resistance to immune therapy. The circle outlines the three steps of the cancer-immunity cycle: (1) Immunogenicity (yellow); (2) T-cell recruitment and (3) activation. Pancreatic ductal adenocarcinoma resistance to immune therapy is due to the combination of several factors: (1) Low tumor immunogenicity, with a low mutation rate and low neaoantigen burden compared to other tumors (*e.g.*, melanoma); (2) low T-cell recruitment and (3) activation: the dense desmoplastic stroma generates high interstitial pressure; this results in poor tumor perfusion and intra-tumor hypoxia, which in turn activates fibroblasts to release immunosuppressive cytokines (*e.g.*, TGFβ, IL-6, CSF1 = "chemical barrier") that lead to the recruitment of immunosuppressive cells (M2 macrophages, TREG, MDSC) and exclusion and anergy of effector T cells. CSF1: Colony stimulating factor 1; IL-6: Interleukin-6; MDSC: Myeloid-derived suppressive cells; TGFβ: Transforming growth factor β; TREG: T regulatory cells.

Research challenges

Rational combinations: Following the failure of CPI monotherapies in PDAC, efforts have been made to develop rational combinations to overcome PDAC resistance to immune therapy. Based on the cancer immunity cycle[25], most of them combine a CPI with another agent aiming to (1) increase tumor immunogenicity; (2) increase TIL number and activity; and/or (3) attenuate immunosuppression in the tumor microenvironment. Combination therapy can employ immune therapy, conventional chemo/radiotherapy, targeted therapy, or vaccine/adoptive T-cell therapy[50,68].

Increasing tumor immunogenicity: Chemotherapeutic agents and radiotherapy may play a dual role by directly killing cancer cells, thus reducing the overall tumor burden and indirectly by releasing pro-inflammatory molecules and tumor-associated antigens (TAA) (*e.g.,* calreticulin, ATP) which, when presented in an immunogenic fashion, may function as *in situ* vaccines to attract and activate T cells (so called "immunogenic death"). Among chemotherapeutic agents used in the PDAC therapeutic armamentarium, platinum-based agents and taxanes are preferential combination partners for immunotherapy because they can induce immunogenic cell death, sensitize tumor cells to immune-mediated destruction and enhance T cell activation[69-71]. Although some investigators have shown that FOLFIRI [folinic acid, 5-fluorouracil (5FU) and irinotecan combination] can be given with vaccines to CRC patients without abrogation of the immune response[72], 5FU and irinotecan have been reported to be more immunosuppressive[73]. Therefore, combining them with an immune therapy may impair the immune-mediated anti-tumor response, and a sequential design for immune therapy after induction chemotherapy using these agents may be more effective.

Tumor vaccines and oncolytic viruses both aim at increasing tumor antigen recognition by the immune system through presentation by dendritic cells[74,75]. Although relatively inefficient as monotherapies, vaccine strategies are currently explored in combination with CPI. GVAX is a granulocyte-macrophage colony-stimulating factor (GM-CSF)-secreting allogeneic PDAC vaccine. It was first evaluated in combination with anti–CTLA-4 therapy[76]. Thirty pre-treated PDAC patients were randomized to receive ipilimumab alone or combined with GVAX. The latter experienced a longer median overall survival (OS) (3.6 mo *vs* 5.7 mo, $P = 0.07$) with no additional toxicity. Furthermore,

the observation that neoadjuvant GVAX was able to induce intra-tumoral tertiary lymphoid structures and upregulate PD-L1 membranous expression in resected tumor samples[30] provided a rationale for its combination with anti–PD-1. This was also supported by preclinical data in mouse models[77] showing an improved survival rate with the combination of GVAX and PD-1 blockade compared to each agent taken individually. In clinical practice, GVAX is associated to cancer vaccine CRS-207 (an attenuated form of *Listeria monocytogenes*) and/or cyclophosphamide (aiming at downregulating TREG) in clinical trials in the adjuvant setting[78]. GVAX/cyclophosphamide therapy is also currently being tested in PDAC in combination with nivolumab (anti–PD-1) alone (NCT02243271, NCT02451982, NCT03161379) or combined to ipilimumab (anti-CTLA-4) (NCT03190265), or with pembrolizumab (anti–PD-1) alone (NCT02648282) or combined to the indoleamine-2,3 dioxygenase (IDO, an enzyme that inhibits T cells proliferation by catalyzing the degradation of tryptophan[79]) inhibitor epacadostat (NCT03006302). Restoring the proliferation and activation of various immune cells, including T cells[80], may potentiate the response to vaccine therapy. Of note, there is also a rationale for combining GVAX with TGFβ inhibitors in preclinical models[77,81]. However, this combination has not reached clinical trials. GVAX, like peptidic "one-size-fits-all" vaccines, has to face the challenges of (1) the unique tumor antigen landscape specific to each patient and (2) the emergence of immune evasion, both of which can compromise patient response to vaccine therapy[82]. Personalized vaccine approaches are expected to partially overcome these issues but their development remains limited by their logistic complexity and high costs[82-84]. Alternatively, oncolytic viruses combine antigen presentation with the induction of a type Ⅰ interferon-γ (IFN-γ) response that potentiates effector T-cell activation[74,75]. Similar to the vaccine approach, the oncolytic virus reolysin was tested in metastatic PDAC in combination with carboplatin and paclitaxel but failed to improve progression-free survival (PFS)[85]. However, a phase Ⅱ study[86] explored the combination of reolysin, pembrolizumab (anti–PD-1) and chemotherapy in 11 patients with pre-treated PDAC and showed antitumor activity with a manageable safety profile. Among the 5 evaluable patients, two had stable diseases (126 and 221 d) and one had partial response lasting more than 6 mo. A phase Ib trial in combination with pembrolizumab and gemcitabine, irinotecan or leucovorin/5-fluorouracil (5-FU) is ongoing (NCT02620423).

Increase TIL recruitment and activity: Most anti–PD-1/PD-L1-based combination trials focus on converting the PDAC non-inflamed (immune-excluded or desert) microenvironment into an inflamed pattern by increasing T cells recruitment and activity.

CPI combination: The association of CTLA-4 and PD-1 antibodies resulted in an improved OS in patients with advanced melanoma compared with each agent used as monotherapy, albeit at the price of increased toxicity with 59% of patients experiencing grade 3 or 4 adverse events (*vs* 21%-28% with monotherapy)[87]. The PA.7 randomized phase Ⅱ trial (NCT02879318) explores the combination of tremelimumab (anti–CTLA-4 mAb) and durvalumab (anti–PD-L1 mAb) with gemcitabine plus *nab*-paclitaxel chemotherapy *vs* chemotherapy alone as a first-line treatment for metastatic PDAC. Co-targeting of other immunomodulatory pathways such as IDO, OX40, CD40, the lymphocyte activation gene 3 protein (LAG3) or T cell immunoglobulin and mucin 3 (TIM3), among numerous candidates, might be as efficient and less toxic than PD-1/CTLA-4 combination[88] but remain to be explored in PDAC patients.

Combination with anti-M2/-MDSC: The CCL2-CCR2 chemokine axis induces the recruitment of immunosuppressive tumor-associated-macrophages (TAM)[89]. A CCR2 inhibitor (PF-04136309) has been tested in combination with FOLFIRINOX chemotherapy in a phase Ib study in patients with borderline resectable/locally advanced PDAC[89]. The objective response rate was 49% and disease control rate reached 97% with a manageable safety profile. Interestingly, ancillary studies showed (1) a decrease in TAM infiltration together with (2) a decrease in circulating monocytes and (3) an increase in bone marrow monocytes in patients treated with the combination, supporting the mechanistic hypothesis of a reduction in intra-tumor monocyte recruitment from the bone marrow[90].

Other inflammatory pathways have been targeted using small molecules or mAb and are currently being explored in clinical trials in combination with CPI based on promising results in mouse models. These include colony stimulating factor 1 receptor (CSF1R)[91] (NCT02777710), IL-6[92], TGFβ (NCT02734160), CCR4 (NCT02301130), CXCR2 (NCT02583477) and CXCR4/CXCL12 (NCT03168139). Nonetheless, similarly to the results obtained following pathway inhibition using tyrosine kinase inhibitors, secondary resistance due to cytokine axes compensation has emerged, leading to disease progression and pleading for combination strategies[93].

Combination with MEK inhibitors: MEK inhibition (MEK-i) was primarily developed in PDAC as a *KRAS* signaling inhibition strategy, given the high frequency of activating *KRAS* mutations in these tumors (> 90%)[94]. MEK-i failed to improve the survival rate of PDAC patients when used as monotherapy or in combination with gemcitabine[94]. However, novel perspectives are opening up for MEK-i as a combination partner with immune therapy. Indeed, MEK-i exerts multifaceted immunostimulatory effects by (1) increasing MHC-I expression and decreasing PD-L1 expression on tumor cells, (2) increasing TIL activity and survival, and (3) decreasing macrophage and MDSC infiltrates[95].

A phase Ib study (NCT01988896) has investigated

the combination of cobimetinib (MEK-i) with atezolizumab (anti–PD-L1) in pre-treated metastatic CRC; durable objective responses were observed in patients with microsatellite stable (MSS)/MSI-low tumors, mostly *KRAS*-mutated, prompting the evaluation of this combination in PDAC in a clinical trial (NCT03193190).

Targeting tumor hypoxia: Likewise, hypoxia-targeting strategies have been tested with disappointing results in combination with gemcitabine[96]. Evofosfamide (TH-302) is a cytotoxic prodrug that is activated under hypoxic conditions, targeting hypoxic tumor areas. It is now being explored as a combination partner for immunotherapy since it can improve tumor tissue oxygenation and subsequently decrease MDSC recruitment and increase effector T cell activity[59,97]. The use of TH-302 with CPI may therefore be effective in restoring a favorable immune environment. A phase Ⅰ trial is underway to study the combination of TH-302 with ipilimumab (anti-CTLA-4) in PDAC, melanoma, head and neck cancer and prostate cancer (NCT03098160).

Targeting fibroblasts and the stromal physical barrier: There have been contradictory reports on the roles of the desmoplastic stroma in PDAC (tumor-promoting *vs* tumor-restrictive effect). CAF elimination using sonic hedgehog inhibitors or genetic strategy for selective depletion of α-smooth muscle actin (α-SMA)-positive cells in transgenic mice resulted in aggressive and undifferentiated tumors with increased vascularization and TREG infiltration, respectively[98,99]. Clinical trials with hedgehog inhibitors in PDAC were negative for any anti-neoplastic activity[100]. Strategies then shifted toward stroma modulation rather than depletion.

Focal adhesion kinase (FAK) is a cytoplasmic tyrosine kinase protein that has been reported to be overexpressed and active in many solid tumors, including PDAC[101]. FAK is expressed by fibroblastic cells as well as tumoral, endothelial and immune cells[101], and its inhibition engenders pleiomorphic effects[102]. In preclinical models, FAK inhibition reduced fibrosis, decreased the amount of tumor-infiltrating immunosuppressive cells, and rendered the previously unresponsive KPC mouse models sensitive to PD-1 blockade[102]. Two phase Ⅰ/Ⅱ studies are underway to verify the benefit of this combination (NCT02546531 and NCT02758587). Other CAF-modulating or anti-fibrotic agents are also under investigation including TGFβ inhibitors (NCT02734160), PEGPH20 (NCT03193190) and vitamin D (NCT03331562) in combination with CPI. In addition, all-trans-retinoic acid (ATRA) (NCT03307148), and BET-inhibitors (NCT02711137) are being explored in combination with chemotherapy.

CAR-T cells: Adoptive cell therapy is a technology that has recently drawn increasing attention. T cells may be engineered to express a chimeric antigen receptor (CAR) in order to target specific tumor antigen[103]. This approach has already proven its effectiveness in B-cell hematological malignancies with T cells expressing CD19 CAR[104,105]. Similarly, mesothelin CAR-T therapy has been proposed in solid tumors[106]. In PDAC, this therapy led to the prolonged survival in a mouse model study[107]. Nevertheless, clinical development of this strategy in solid tumors is hampered by (1) its limited efficacy in comparison with the results seen in hematological malignancies; (2) high level of toxicity, including life-threatening immune adverse events (neurotoxicity and cytokine release syndrome); and (3) costs and logistics to be deployed on a large patient population. Next generation CAR T-cells are currently being developed to overcome these challenges[108].

CONCLUSION

Rethink current clinical trial approaches

Besides exploring new therapeutic avenues, it is also necessary to rethink the design of clinical immune therapy trials targeting PDAC. The clinical trial design tends to shift from traditional phase Ⅰ to Ⅲ development plan toward a signal detection strategy in multiple patient cohorts. In the context of an increasing number of clinical trials, there is a need to identify the most relevant combinations among the numerous candidate agents. Development of new preclinical models closer to the complex *in vivo* conditions should significantly improve the predictive value for therapeutic agent testing and guide the selection of the most active combinations for evaluation in clinical trials.

Second, the examples of MEK-i, vaccines, evofosfamide or TGFβ inhibitors show that it may be worth giving a second chance to some molecules that were found inactive as monotherapy.

In addition, patients with heavily pre-treated, progressive, advanced PDAC are not good candidates for immune therapy and this may partially account for failure of previous studies. These patients should possibly be excluded from immunotherapy clinical trials. Alternatively, positioning immune therapy as maintenance strategy following a course of induction chemotherapy (*e.g.,* with FOLFIRINOX) seems to present several advantages: (1) It allows the identification and exclusion of patients with rapid tumor progression; (2) such a treatment may have induced immunogenic cell death and sensitized the tumor to CPI; and (3) given that induction chemotherapy was not interrupted due to inefficacy, it could be reintroduced at disease progression. Taken together, these elements support the development of immune therapy as maintenance therapy in patients with controlled disease.

Finally, there is a critical need for predictive biomarker identification in order to guide patient selection for immune therapy and to stratify the randomization. Meanwhile, it is necessary to assess the predictive value of already available PDAC molecular classifications in the ancillary studies of ongoing clinical trials[109-112].

Future directions

PDAC is resistant to CPI monotherapy due to its unfavorable non-immune inflamed microenvironment. A better understanding of the biological mechanisms underlying PDAC immunosuppression may pave the way to innovative and promising strategies. Given the key role of the team hypoxia-TGFβ-CAF-M2/MDSC, the development of rational combinations of immunotherapy targeting these pathways and cell populations to increase intra-tumor recruitment and activation of T cells is coherent. To achieve this, we will have to reconsider inactive molecules in monotherapy, optimize the position of immunotherapy in the therapeutic sequence and develop new preclinical models to better predict therapeutic efficacy.

Furthermore, an improved understanding of the mechanisms of sensitivity and resistance to immunotherapy has revealed the increasing complexity in the tumor antigens, TIL, TREG, and MDSC landscape[113]. For instance, (1) anti-inflammatory and pro-inflammatory cytokines have counter balancing activities; (2) biological effects may be different between primary and metastatic tumor sites as illustrated by dissociated responses; (3) hypermutated tumors are more likely to respond to but also to develop resistance to CPI[114]; and (4) the immune therapy response is also dependent on the patient microbiota[115,116] and genetics[117]. Mechanisms of action of CPI remain yet to be fully elucidated. The collaboration between clinicians and researchers will be the cornerstone of future progress in this field.

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MINIREVIEWS

Liver transplantation and multivisceral transplantation in the management of patients with advanced neuroendocrine tumours

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Abstract

Orthotopic liver transplantation (OLT) represents a generally accepted albeit somewhat controversially discussed therapeutic strategy in highly selected patients with non-resectable hepatic metastases from neuroendocrine tumours (NET). Whilst there are some exclusion criteria, these are not universally followed, and the optimal set of inclusion parameters for deeming patients eligible has not yet been elucidated. This is due to heterogeneity in the study populations, as well differing approaches employed and also divergences in selection criteria between centres. Recent data have suggested that OLT may represent the most efficacious approach in terms of overall and disease-free survival to the management of NET metastatic to the liver when conducted in accordance with the modified Milan criteria. Therefore, a consensus set of selection criteria requires definition to facilitate stringent and fair allocation of deceased-donor organs, as well as consideration for living-donor organs. In the context of classically non-resectable metastatic tumour bulk, multivisceral transplantation with or without the liver may also be indicated, yet experience is very limited. In this review, we discuss the diagnostic work-up of patients in whom the aforementioned transplantation approaches are being considered, critically analyse the published experience and also anticipate future developments in this field, including a discussion of immediate and longer-term research priorities.

Key words: Neuroendocrine; Transplantation; Metastases; Liver; Multivisceral

Core tip: Liver transplantation is a generally accepted option in selected patients with advanced neuroendocrine tumours metastatic to the liver. Outcomes may be favourable in exquisitely selected patients, yet the optimal selection criteria have not yet been elucidated. Multivisceral transplantation is valid but rarely utilised, for example, in cases of metastatic bulk threatening gut

vascular supply.

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INTRODUCTION

Representing an increasingly prevalent class of neoplasms, neuroendocrine tumours (NET) display protean clinical manifestations, and those arising from the lung, pancreas and bowel possess a particular proclivity for metastasis to the liver. Up to 90% of small bowel NET display evidence of at least nodal metastasis at initial diagnosis[1], and the incidences of liver metastases (LM) in small bowel and pancreatic NET treated at specialist centres range between 67%-91% and 28.3%-77%, respectively[2,3]. The liver is the sole location of distant oligo-metastatic disease in approximately half of all NET[4] and their presence has markedly detrimental impact on the long-term survival of NET patients, thus conferring great significance on the management of neuroendocrine liver metastases (NELM)[5-7].

Therapeutic strategies for NELM may incorporate surgical approaches, *i.e.,* resection with curative or palliative intention, peptide receptor radionuclide therapy, liver-directed trans-arterial or percutaneous treatments and medical therapies[8]. Hepatic surgery is the only approach offering potential cure, and resection of liver deposits if attainable has classically been held as the first-line modality conferring the best survival outcomes[8]. However, cure is rarely realised even with complete elimination of the hepatic tumour burden as patients almost invariably develop recurrent disease, and resection should be regarded in most to be a palliative endeavour. Under the premises of complete resection of the primary tumour and loco-regional lymph node metastases , the radical approach of total extirpation of the liver with unresectable NELM in the context of orthotopic liver transplantation (OLT) has re-gained attention as outcomes continue to improve. In fact, stringently selected patients undergoing OLT may actually attain the most favourable survival outcomes, based on recent data from Mazzaferro and his group following the modified Milan ('Milan NET') criteria[9]. However, there is great divergence in the selection criteria followed at different centres, and a recent systematic review of retrospective case series calculated a median overall survival at 1-, 3-, and 5-years of 89%, 69% and 63%, respectively[10].

Multivisceral transplantation (MVT) with or without the liver (*i.e.*, modified [M]MVT)[11] is a seldom utilised approach for highly selected patients with extensive metastatic burden, either in those with pancreatic head tumours and LM[12], or potentially some patients with no LM but extensive mesenteric lymph node metastases threatening vascular supply to the gut by encasement of mesenteric vessels[13,14]. Again, recent data suggest improving outcomes over time with such approaches involving intestinal allografts[15] and therefore these could be more widely utilised in the near future.

In this review, we provide an overview of the diagnostic work-up of patients with NELM being considered for transplantation, specifically the power of both functional and morphological imaging in patient selection. Thereafter, we provide a critical analysis of the reported outcomes from OLT and MVT/MMVT and conclude with discussion of future perspectives in this burgeoning field.

PRE-TRANSPLANT EVALUATION – PATIENT SELECTION

Liver transplantation may be offered to patients with metastases of low- or intermediate grade (G1/2) NET (Ki67 of < 20%[16]) confined to the liver without extra-hepatic metastases, unless these are themselves resectable[8]. Up to 80% of NELM display diffuse multifocal and bilobar spread, and are therefore not amenable for standard resections with curative attempt[17]. In patients with non-miliary metastases but nevertheless conventionally non-resectable hepatic disease, advanced surgical procedures such as ALPPS may be considered to offer chance of resection *via* a two-stage approach[18,19]. Accordingly, meticulous selection of patients with advanced NET for transplantation approaches relies on the use of high quality imaging strategies to accurately depict disease burden, with emphasis both on the distribution of disease within the liver, but especially also possible extra-hepatic deposits as these could render a patient ineligible for transplantation (Figure 1A-C). Morphological and functional imaging modalities have important roles in the evaluation of NET and their metastases.

As most NELM are hypervascular, computed tomography (CT) imaging must include hepatic arterial phases[20]. Furthermore, diffusion-weighted magnetic resonance imaging (DW-MRI) should be systematically performed in any evaluation of NELM as it possesses the highest specificity of all MRI phases, even in tumours < 1 cm in size[21].

Functional imaging with positron emission tomography (PET) using 68-gallium radiolabelled DOTA peptides combined with CT (*e.g.*, ^{68}Ga-DOTATATE or ^{68}Ga-DOTATOC PET/CT) represents the gold standard approach in G1/G2 NET as it may detect lesions that morphological imaging modalities cannot, as well as those not identified by somatostatin-receptor scintigraphy with ^{111}In-conjugated radiopharmaceuticals[21-23]. Imaging with ^{68}Ga-DOTA PET/CT detects NELM with a sensitivity between 82%-100%, a specificity of

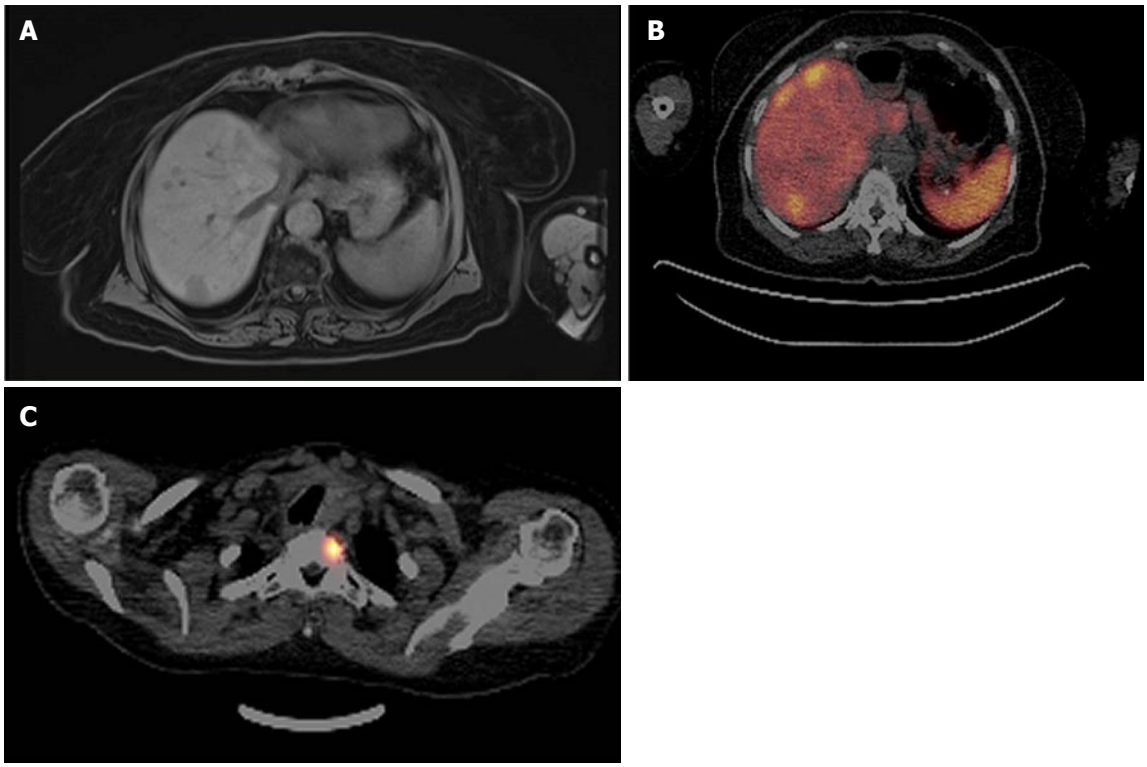

Figure 1 Multimodality imaging in a patient with neuroendocrine liver metastases considered for transplantation. A: Magnetic resonance imaging of the liver in a patient with hepatic metastases from a small bowel neuroendocrine tumour. This patient underwent resection of the primary tumour, and then a left hepatectomy. Following post-hepatectomy lanreotide, peptide receptor radiotherapy and also selective internal radiotherapy for recurrent hepatic metastases, this patient was considered for orthotopic liver transplantation. There was no extra-hepatic disease on conventional cross-sectional imaging. B: ^{68}Ga-DOTATATE PET/CT in the same patient. Multiple foci of increased avidity are demonstrated within the liver that were not appreciated on magnetic resonance imaging. C: Radiotracer uptake corresponding to one of multiple bone metastases. According to standard criteria, these would exclude this patient from orthotopic liver transplantation.

67%-100%, and also detects extra-hepatic disease with a sensitivity of 85%-100% and a specificity of 67%-90%[23]. In fact, a major proportion of the power of ^{68}Ga-DOTA PET/CT in terms of surgical selection is in its ability to identify extra-hepatic disease that is capable of altering clinical strategies[24,25], which is especially relevant when considering visceral transplantation.

Novel radiotracers for PET/CT, such as those using 64-copper have shown promising results comparable to ^{68}Ga-DOTA , although they are not in wide circulation as of yet[26]. The archetypal oncological radiotracer ^{18}F-FDG is widely used in the imaging of adenocarcinomas, and there is increasing evidence to support its implementation in the radiological work-up of NET patients alongside ^{68}Ga-DOTA PET to assess the metabolic activity of tumours which correlates with disease aggressiveness and prognosis[27]. However, one may argue that there is limited (if any) role of ^{18}F-FDG PET/CT in NET patients as part of pre-transplant work-up as lower-grade disease is the *sine qua non* for consideration of this approach. Additional radiotracers have also been assessed in cohorts of NET patients, especially in European centres, specifically ^{18}F-DOPA and ^{11}C-5-hydroxytryptophan[28,29]. However, experience with these tracers is limited, and can at present only be recommended as part of investigative studies, or as an adjunct to lessen radiological uncertainty when there are inconclusive findings with 'standard' functional imaging.

Alongside detailed radiological depiction of disease status, patient functional status and relevant co-morbidities must also be evaluated in the overall assessment of patients being considered for transplantation. Carcinoid heart disease (CHD) manifests as fibrous endocardial thickening involving cardiac valves and sub-valvular apparatus, particularly in the right heart. It has an incompletely elucidated aetiology but is presumed to be linked to excessive circulating vasoactive substances secreted by NET, and exerts considerable morbidity and mortality in NET patients. Transthoracic echocardiography is the gold-standard modality for assessment of cardiac function in patients suspected of having/at risk of CHD[30]. Furthermore, untreated CHD is an accepted contraindication for OLT, and should be treated before OLT, or even any hepatic surgery is planned[30].

Patients with advanced NET considered for transplantation require extensive evaluation. This includes assessment of their anaesthetic risk and co-morbidity profile, including specific emphasis on the presence (and if applicable, treatment of) carcinoid heart disease, which is a contraindication to transplantation. Radiological evaluation of disease should include CT (hepatic arterial phase), MRI (especially DW-MRI) and if available, ^{68}Ga-DOTA PET/CT. The latter is essential in

Table 1 Results from liver transplantation in selected registry reports, multicentre series and recent single centre series

Ref.	Year	Study type/setting	Total patients		Overall survival (%)						Disease-free survival (%)					
					1 yr	2 yr	3 yr	4 yr	5 yr	10 yr	1 yr	2 yr	3 yr	4 yr	5 yr	10 yr
Nobel *et al*[58]	2015	Registry (UNOS)	120		87		69		63							
Le Treut *et al*[38]	2013	Registry (ELTR)	213 (6 MVT)	*Overall*	81	73	65	55	52		65	49	40	33	30	
				ELTR score 0-1					79						57	
				ELTR score 2-3					38						19	
Nguyen *et al*[40]	2011	Registry (UNOS)	184	*Overall*	79.5		61.4		49.2							
				Post-MELD	84.7		65		57.8							
Gedaly *et al*[37]	2011	Registry (UNOS)	150 (13 MVT)		80		64		48		77[1]		50[1]		32[1]	
Sher *et al*[51]	2015	Multicentre series (United States)	85		83		60		52							
Mazzaferro *et al*[9]	2016	Single centre series (Italy)	42						97.2	88.8					86.9	86.9
Bonaccorsi-Riani *et al*[59]	2010	Single centre series (Belgium)	9		88		77		33		67		33		11	
Olausson *et al*[50]	2007	Single centre series (Sweden)	15 (5 MVT)						90				70		20	
Van Vilsteren *et al*[60]	2006	Single centre series (United States)	19		88						80					
Frilling *et al*[61]	2006	Single centre series (Germany)	15 (1 MVT)		78.3				67.2		69.4				48.3	

[1]Calculated from 83 patients. UNOS: United Network for Organ Sharing (United States); ELTR: European Liver Transplant Registry; MELD: Modified end-stage liver disease score; MVT: Multivisceral transplantation.

patients considered for liver transplantation as it enables the best opportunity for the depiction of extrahepatic disease which could invalidate this form of approach. As it represents the gold-standard imaging modality in NET, ^{68}Ga-DOTA PET/CT is also most useful in patients considered for intestinal/multivisceral transplantation.

ORTHOTOPIC LIVER TRANSPLANTATION

Curative (R0) resection of NELM may be associated with the most favourable survival outcomes in reported retrospective series however is subject to significant limitations[8,31-33]. First, approximately 80% of patients with NELM will not be eligible for this approach due to the anatomical distribution of hepatic disease burden abrogating the feasibility of radical surgical tumour elimination[17]. Second, to what extent R0 resection is actually associated with favourable outcomes cannot be confidently assessed given that studies are retrospective and thus outside the auspices of randomised trials, and that whilst patients are highly selected, the selection criteria themselves are often very poorly defined, if at all[34]. Essentially, the effects of favourable tumour biology and favourable patient characteristics, such as co-morbidity profiles are impossible to disentangle from the reported outcomes due to this selection bias. Third, even in patients undergoing hepatectomy/other hepatic resection with curative intent, vertiginous rates of recurrence are clearly recognised[8,33], to the extent that disease recurrence should not only be considered, but actively expected. The juxtaposition of favourable overall-survival against starkly poor disease/recurrence-free survival in hepatic resection is attributable to most likely the presence of undetected micro-metastases that given the relative indolence of NET, clinically manifest over a protracted period of time. Current gold-standard imaging modalities understage disease burden by 50% when compared with meticulous pathological examination[35], thus explaining the clinical reality that resection with curative intent is almost always a palliative endeavour, albeit an excellent one in terms of significant improvement in the duration of patient overall survival.

Therefore, OLT represents an attractive paradigm for radical therapy of NELM, insofar as total hepatectomy with subsequent transplantation theoretically offers complete resection of both macro- and micro-metastatic disease burden at a single time-point. This approach is heavily debated and rarely utilised (just over 700 patients)[36], and represents only 0.2%-0.3% of all liver transplants recorded in US/European liver transplant registries[37,38]. Table 1 summarises recent published experience from selected series.

There is growing evidence to support consideration of wider implementation of OLT in NET. However, major obstacles include the already heavy demands on deceased-donor livers for non-malignant conditions and also HCC, as well as the limited use of living-donor liver transplantation (LDLT) outside of Asia, where LDLT accounts for up to 60%-90% of all liver transplant activities in some countries[39]. The use of LDLT of course introduces complex ethical considerations, such as risks of morbidity and perhaps even mortality to the healthy donors.

Results with orthotopic liver transplantation

A recent comprehensive systematic review of Moris *et al*[10] identified 64 studies for inclusion, 4 of which represented registry reports (which were described narratively), and 57 were single-centre reports. Registry

reports did not uniformly document the primary tumour site in transplanted patients, but cumulative analysis of single-centre studies identified the pancreas as the primary tumour derivation in the majority of patients (53.4%) with the ileum the second most common (23%). However, only 3 studies described the histologic type of these primary tumours. The majority of patients presented with synchronous hepatic disease, and most received pre-transplant therapy with medical modalities (hormone-based or chemotherapy), resection of primary tumour or NELM resection. Only approximately 5.6% of patients did not undergo any pre-OLT treatment. Given the large number of heterogeneous studies, rates of concomitant primary tumour resection and OLT were not reported, nor were the comparative survivals between patients receiving pre-OLT treatment or not. Regarding immunosuppression therapy utilised, no large cohort studies discussed this. With regards to the long-term outcomes with OLT, 1-, 3-, and 5-year OS was 89%, 69% and 63%, respectively. Recurrence after LT ranged between 31.3-56.8%. There was no clear information regarding the radiological modalities used in pre-transplant assessment, nor in follow-up; therefore one could speculate that recurrence may in truth be higher if ^{68}Ga-DOTA PET/CT was not used during follow-up.

The review of the United Network for Organ Sharing (UNOS) database by Gedaly *et al.* reported 150 liver transplants performed for metastatic NET (of a total of 87280) between October 1988 and January 2008[37]. Thirteen of these patients received more than one organ (see later), and the overwhelming majority (91.3%) underwent LT using organs from deceased donors. The tumour histology/functional status was not uniformly reported, with 46.7% of cases documenting 'unspecified NET'. Gedaly and colleagues calculated 1-, 3-, and 5-year OS rates of 81%, 65% and 49%, respectively for patient undergoing OLT. Recurrence information was available for 83 patients, and 1-, 3-, and 5-year DFS rates were 77%, 50% and 32%, respectively. There was no significant difference observed in survival in patients older or younger than 55years, however there was a significant improvement in 5-year survival in patients undergoing transplantation after the 67day median wait-time versus those transplanted earlier (63% *vs* 36%). Lastly, an interesting comparison was drawn between OS of patients undergoing OLT for NET and HCC (n = 4693) which failed to identify any significant difference.

Another study from the UNOS database encompassing a wider time-frame (1988 to March 2011) and 184 patients with metastatic NET focussed on the effect of the introduction of the model for end-stage liver disease score/paediatric model for end-stage liver disease (MELD/PELD) scores in 2000 on OLT outcomes[40]. Overall survival rates for the entire NET cohort at 1-, 3-, and 5-years were 79.5%, 61.4% and 49.2%, respectively. In contrast to the aforementioned UNOS database study[37], these rates were significantly lower than those observed in patients with HCC, or those undergoing LT for non-malignant indications in the same time period (85.8%, 71.1% and 60.6%; 85.2%, 78.3% and 73%). Seventy-four OLT for NET occurred prior to MELD/PELD introduction, and these patients had significantly worse survival outcomes compared to those transplanted following MELD/PELD implementation. Pursuant to this, when only the LT for NET occurring after 2002 were considered, there were no significant differences between overall survival when compared to HCC (84.7% *vs* 88%; 65% *vs* 74.3%; and 57.8% *vs* 64.4%), although patients transplanted for non-malignant indications fared significantly better (87.1%, 79.5% and 73.7%).

The largest series yet reported is the analysis of the European Liver Transplant Registry by Le Treut *et al*[38]. Their retrospective analysis over a 27-year period identified 213 patients receiving LT for one of 3 classes of indication: hormonal syndrome/symptoms (17%), tumour bulk (24%), or 'oncological' (54%). The LM were synchronous in 119/213 cases, and the median interval between diagnosis of LM and LT was 25 months (1-149). Prior to LT, 83% of patients underwent surgical therapy targeting the primary tumour (n = 158) or LM (58); these included 23 cases of major hepatic resection (10.8%). In terms of non-surgical treatment, there were 161 instances of 'chemotherapy' (76%) including somatostatin analogues in 63 patients, and trans-arterial chemoembolisation in 76. The 3-mo post-operative mortality was 10%, with early re-transplantation, upper abdominal exenteration, splenectomy, operative duration > 10 h, R1/R2 resection margin, hepatomegaly and surgery in addition to LT identified as significant arbiters of this. Regarding survival, the median OS post-LT was 67months, with 1-, 3-, and 5-year overall survival rates of 81%, 65% and 52%, respectively. Disease-free survival rates at the same intervals were 65%, 40% and 30%, respectively. There were no associations between long-term survival and three age cut-offs, nor time between diagnosis and LT. However, poor prognosis generally was associated with major resection in addition to LT, poorer tumour differentiation and hepatomegaly. Furthermore, as the authors identified improved outcomes in those transplanted after 2000 (n = 106, 59% OS *vs* 46% prior to this), multivariate analyses were utilised to develop a 4-point prognostic scale in which the presence/absence of hepatomegaly, age > 45, or their undergoing major resection with LT were considered/'scored'. Patients with 0/1 of these factors demonstrated 5-year OS and DFS of 79% and 57%, respectively, whereas patients with 2/3 of these predictors had 5-year OS and DFS of 38% and 19%, respectively.

Clearly, these larger studies are limited by the heterogeneity of included patients. This has effects on the divergent adverse prognosticators identified[41]. Furthermore, the selection criteria utilised are usually very poorly documented. An exception to this is the

Table 2 Comparison of published selection criteria for liver transplantation in neuroendocrine liver metastases, and cirrhosis with hepatocellular carcinoma

Criteria and context	Parameters
Milan NET criteria[42]	Age < 60
Neuroendocrine liver metastases	G1/G2 tumour grade
	Primary tumour drained by the portal venous system
	Metastatic involvement limited to the liver
	Hepatic tumour burden not > 50%
	Six months of no tumour progression
Milan criteria[62]	Single tumour ⩽ 5 cm
HCC and cirrhosis	Or, ⩽ 3 tumours each ⩽ 3 cm in size
	No macrovascular invasion
UCSF criteria[63]	Single lesion ⩽ 6.5 cm
HCC and cirrhosis	Or, 2-3 lesions ⩽ 4.5 cm each, with total tumour diameter ⩽ 8 cm
	No macrovascular invasion
Navarro criteria[64]	Single lesion ⩽ 6 cm
HCC and cirrhosis	Or, 2-3 lesions ⩽ 5 cm each
	No macrovascular invasion
Valencia criteria[65]	1-3 lesions ⩽ 5 cm each, total tumour dimeter ⩽ 10 cm
HCC and cirrhosis	No macrovascular invasion
'Up-to-7' criteria[66]	Number of tumours + size of tumours (in cm) ⩽ 7
HCC	No microvascular invasion

HCC: Hepatocellular carcinoma; G: Grade.

recent data from Milan, which have detailed impressive outcomes from patient selection using their 'Milan NET' criteria[42]. Table 2 compares the Milan NET criteria for NELM and also documented transplantation criteria for HCC, including the original Milan criteria applicable only to HCC.

In their most recent report of a prospective series, Mazzaferro, *et al*[9]. reviewed 88 patients referred for consideration of OLT, of which 42 were offered transplant. Forty-six patients either had waiting-list conditions that precluded transplant consideration, or refused transplantation. In those undergoing OLT, the median OS was not attained, whilst 5-year and 10-year OS rates were 97.2% and 88.8%, respectively. Rates of disease progression in those receiving OLT were 13.1% at 5- and 10-years, *i.e.,* all recurrence/progression occurred within the first 5 years of follow-up. Contrastingly, 5-, and 10-year OS rates in those not undergoing OLT were 50.9% and 22.4%, respectively. Follow-up comprised CT or MRI every 3-4 mo, with Octreoscan, ^{68}Ga-DOTA PET/CT or ^{18}F-FDG PET/CT only used when morphological imaging/chromogranin assays were suspicious for recurrence. There was no clear documentation on how many patients underwent each of these tumour-targeted imaging modalities, nor what their specificities were for recurrent disease.

Although these survival outcomes certainly appear to be the most favourable encountered in the literature pertaining to therapy of NELM, these results must be considered with due diligence as by their nature, such studies possess important inherent bias, similar to those expressed by series of hepatic resection. Whilst tumour burden did not differ between the transplanted and non-transplanted groups, patients not undergoing transplant were significantly older than those that did (median 55.5 years *vs* 40.5 years), had higher T stages of the primary tumour (69.5% T3/4 *vs* 54.8% T3/T4), had higher WHO grade, and underwent less locoregional therapy including liver resection, transarterial chemoembolisation (TACE) or peptide receptor radionuclide therapy (PRRT) (73.9% of the non-transplant group received none *vs* 57.1% of the transplanted group). Lastly, the earlier discussed prognostic score as developed by Le Treut *et al*. was 0 or 1 in 52.4% and 35.7% of transplanted patients, respectively. Evidently, patients undergoing OLT are incredibly highly selected and thus the extent to which positive outcomes can be attributed to appropriate OLT 'itself' rather than favourable patient/tumour biology is unclear. It may be possible that a considerable proportion of transplanted patients would be candidates for hepatic resection. Nevertheless, at face value, these results with the Milan NET criteria appear favourable in the context of an 86.9% 10-year DFS.

Neoadjuvant and adjuvant therapy

There are no significant differences between post-transplant immunosuppression therapy for NELM and HCC. Consideration of neoadjuvant and adjuvant concepts should be incorporated into the multidisciplinary discussion of patients evaluated for possible transplantation. Recurrence rates post-OLT in general range between 31.3%-56.8%[10]. A consensus is yet to be established regarding such approaches, however one may speculate that pre-OLT PRRT, or the use of post-transplant somatostatin analogues could be useful given their anti-proliferative effects as documented in randomised clinical trials[43,44]. These methods could theoretically downstage/control disease prior to transplantation, or retard the development of recurrent

micro-metastases. An additional consideration could be the use of mammalian target of rapamycin (mTOR) inhibitors such as everolimus, which has documented anti-proliferative effects on NET in clinical trials[45] , and also serve immunosuppressive functions with the advantage of exerting no nephrotoxic effects[46,47]. Pre-transplant cytotoxic chemotherapy does not have an established role – indeed, NET in general exhibit a low response rate to such treatment, and the effects of cytotoxic agents appear limited to advanced pancreatic NET[8].

Recent data suggest that OLT is a promising therapeutic option in metastatic NET and may be associated with favourable long-term survival outcomes. It should be used when hepatic disease is controlled, after the resection of the primary tumour, and not as a 'last resort' intervention. In addition, concomitant major resection should be avoided if possible at the time of transplant. Carcinoid heart disease is an accepted contraindication. However, OLT patients present a highly selected cohort, especially those transplanted in accordance with the Milan NET stipulations. The optimal selection criteria require definition, and reports of OLT should adhere to a number of reporting standards (see discussion). The role of neoadjuvant and adjuvant concepts in liver transplantation for NELM needs to be defined to reduce disease recurrence. Outcomes from OLT were initially poor, but have considerably improved as a result of refined immunosuppression regimens, surgical technique and patient selection. In the modern era, outcomes with OLT for metastatic NET are not statistically dissimilar to those encountered in HCC.

INTESTINAL AND MULTIVISCERAL TRANSPLANTATION

Intestinal transplantation (IT) has gained acceptance as a standard therapeutic strategy in patients with intestinal failure failing rehabilitation, diffuse portal thrombosis or other intra-abdominal catastrophe, but has also been performed in patients with non-resectable, slow-growing tumours encasing the mesenteric root as this threatens the vascular supply to the gut[15,48,49]. Transplantation of the intestines may be within the context of simultaneous transplantation of the stomach, duodenum, pancreas and small bowel with (multivisceral transplantation, MVT) or without the liver (modified MVT, MMVT)[11]. Experience with this radical approach in neuroendocrine tumours is incredibly limited to either case reports or to small numbers within cohorts composed predominantly of patients undergoing OLT[13,37,38,50]. In this setting, patients either have pancreatic head tumours, and/or bulky metastatic load within the small bowel mesentery.

Less than 20% of all NET patients undergoing liver transplantation also receive additional organs – in the aforementioned systematic review of Moris *et al*[10] only 5.7% of transplants (16/279) outside the largest registry reports/multicentric series receive a multi-organ allograft. The multicentre series of Sher *et al*[51] included 17 patients (total 85, 20%) undergoing a multivisceral transplantation and reported overall survival rates at 1-, 3- and 5-years of 81%, 40% and 40%, respectively. These were lower than those undergoing OLT, however not significantly so. Thirteen of the 150 patients reported by Gedaly *et al*[37] (8.7%) received additional organs alongside the liver, however the survival data specifically for this sub-set of patients was not clearly detailed as the authors merely stated that on inclusion of MVT cases, the cohort OS data did not change significantly. Lastly, the published data from Nordic centres have described a 2-year overall survival of 67% in 6 patients with pancreatic head NET that underwent intestinal transplantation within a multivisceral graft, which was not inferior to the outcomes from those transplanted for intestinal failure[12].

Clearly, reports of IT/MVT/MMVT in NET are limited by: (1) The small numbers of patients transplanted; (2) the inconsistent quality of outcome reporting and selection criteria in publications; and (3) the inclusion of multiple indications in single publications (often including non-malignant indications).

Nevertheless, as outcomes continue to improve for IT/MVT/MMVT, one may anticipate a cautiously managed expansion of the number of patients with advanced NET being considered for and undergoing such procedures. As with OLT, emergent concepts will include the optimisation of patient selection criteria, as well as innovative neoadjuvant/adjuvant concepts to abrogate disease recurrence and monitor for allograft dysfunction. For example, recent case reports have detailed the use of everolimus post-MVT in 2 NET patients in attempts to suppress recurrence whilst avoiding the nephrotoxicity of calcineurin inhibitors[52], as well as the use of PRRT to stabilise disease prior to MMVT which also included simultaneous transplantation of a sentinel skin flap from the organ donor to aid monitoring of rejection and tailoring of immunosuppression regimens[13].

Intestinal/multivisceral/modified multivisceral transplantation has been utilised in a very small number of patients with advanced NET worldwide. Case series tend to be small yet highly heterogeneous in terms of patient inclusion, and outcome reporting is of varying quality. Nevertheless, innovative approaches continue to be described in the setting of such advanced surgical procedures.

CONCLUSION

For patients with well-controlled, G1/G2 neuroendocrine tumours, transplantation approaches may be valid therapeutic strategies in those with classically non-resectable metastases confined to the liver (OLT) and/or bulky mesenteric tumour load threatening the vascular supply to the gut (IT/MVT/MMVT). It is generally advised that the primary tumour and any attendant locoregional lymph node metastases be resected prior to undergoing OLT, and there is a suggestion that longer

wait times/observance period prior to transplant to monitor for disease stability, although this is not based on high-quality evidence[9,36]. It is debatable if small volume bone metastases are necessarily a contra-indication to transplantation given that they may be well-controlled with PRRT.

Patients are stringently selected in accordance with a mixture of criteria that are either well defined, or barely documented. As with the NET clinical arena in general, the majority of data available to inform modern clinical practice is derived from retrospective case series of varying quality in their reporting. Prospective studies and randomised clinical trials of surgical treatment for NET are logistically challenging given their relative rarity and relative indolence requiring prolonged follow-up, even before considering the difficulties in randomisation of surgical therapy. Transplantation approaches in NET are subject to the same difficulties. Decision making such as expanding the criteria/exceptions of transplant co-ordinating institutions to include NELM will rely on sound identification of patients most suitable for receiving donated organs which in turn can expect the best outcomes. This is mandated in the context of limited yet heavily demanded availability of deceased donor organs and also limited use of LDLT outside of Asia. Therefore, at least in the short-to-medium terms, such decision making must be based on analyses of the currently available data which is mostly of a retrospective nature. Collaborations such as registries and inter-institutional initiatives will enable statistical analysis of ever-larger pooled patient cohorts. Going forward, the non-mutually exclusive NET and surgical communities must recognise the shortcomings thus far experienced in data reporting in order to improve current and future data collection for use in novel informative projects.

In order to counteract the previously discussed deficiencies in data reporting and also facilitate inter-centre collaboration in the analysis of larger cohorts, we propose that each of the following be documented at an individual patient level within institutional databases, and be available to collaborators, notwithstanding ethical approval for the secure sharing of such data:

(1) Indication for transplantation and timing – time between diagnosis and transplantation, duration of disease stability prior to OLT/MVT.

There is a need to clearly distinguish at which point during the patient journey that the best outcomes may be attained. Patients undergoing OLT when disease is controlled with therapy are posited to derive true benefit with excellent survival. Whilst it is suspected that patients undergoing OLT/MVT as an ultima ratio approach will have poorer outcomes, *i.e.* marginal life gains, this needs to be categorically confirmed and also judiciously analysed as a possibly legitimate 'salvage' option.

Reports suggest that the observation of tumour behaviour for 6 months to ensure disease control is associated with preferable outcomes in OLT. This needs to be clearly documented in larger numbers. Such data also add to the temporal treatment trajectory of individual patients, which may be complex as transplant patients are often heavily 'pre-treated'. Clear comparisons will only be valid when results are interpreted in the context of the 'patient journey'.

(2) Clinicopathological characteristics – especially age at transplantation, Ki67 index, hepatic tumour burden (if applicable), clinical syndromes, grade and differentiation of primary tumour and metastases, disease stage (including other metastatic sites and treatment for these), surgical histopathology results (margin and lympho/vascular invasion) and patient co-morbidities.

Optimised selection criteria in the short-to-medium term will likely be developed by multivariable analyses of individual-level data accrued from disparate centres, and clinicopathological characteristics are often reliable arbiters of tumour behaviour and thus patient outcomes. Therefore, clear documentation of parameters that are potentially predictive/prognostic in nature is essential.

(3) Selection criteria – *e.g.* compliance with Milan NET criteria, or other institutional protocols; imaging modalities and patient-specific parameters for disease assessment.

As aforementioned, selection criteria for surgical intervention are typically very poorly documented, confounding the collation and interpretation of multi-centric data. Whilst the Milan-NET criteria are clearly followed in its respective centre, whether or not alternative protocols are used versus collective multidisciplinary decision making should be documented.

(4) Use of neoadjuvant/adjuvant concepts: Despite excellent results from one centre that does not appear to have utilised post-operative prophylaxis against disease recurrence, whether or not such strategies have been/should be employed in other centres has not been documented clearly. As previously discussed, medical therapies with anti-proliferative/anti-tumour effects could theoretically be useful in disease stabilisation prior to transplant, or to reduce the risks of post-transplant recurrence. This must be clearly delineated from pre-transplant treatment and treatment for post-transplant recurrence. The use of such concepts may be included in multivariable analyses to examine for associations between their utilisation and outcomes (or lack thereof/thereon).

Lastly, it is becoming increasingly clear that multifactorial assessment of neuroendocrine tumour characteristics have tangible benefits in not only prognostication[53], but also detection of recurrence[54,55] and prediction of response to treatment[56]. Novel markers developed from 'omics'-based technologies, such as the multi-analyte NETest are able to

predict outcomes from PRRT and also disease recurrence, and therefore possibly offer improved selection and impact follow-up decisions[56,57]. Precise molecular definition of patient-specific neuroendocrine tumour biology may also have ramifications on patient selection for surgery or transplantation, as well as monitoring for detection of recurrence possibly before lesions are detectable on imaging. Such techniques should also be investigated within the remit of transplantation for advanced NET.

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MINIREVIEWS

Characteristics and predictors of gastric cancer after *Helicobacter pylori* eradication

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Abstract

Helicobacter pylori (*H. pylori*) eradication can reduce gastric cancer. However, gastric cancer still develops after eradication, and cases who received eradication therapy are increasing. In this study, we have reviewed the characteristics and predictors of primary gastric cancer developing after *H. pylori* eradication. In terms of the characteristics, endoscopic, histologic, and molecular characteristics are reported. Endoscopically, gastric cancer after eradication is often depressed-type and shows a gastritis-like appearance, which sometimes makes the diagnosis difficult. Histologically, most gastric cancer after eradication is intestinal type, and non-neoplastic epithelium, also called epithelium with low-grade atypia, is frequently seen over the tumor, which is presumably the cause of the endoscopic gastritis-like appearance. As for molecular characteristics, some markers, such as Ki67, MUC2, and Wnt5a expression, are lower in cancer from patients in whom *H. pylori* has been eradicated. In terms of predictors, several Japanese studies have reported that severe endoscopic atrophy at eradication is a risk factor for gastric cancer development. Histologic intestinal metaplasia, especially in the corpus, and long-term use of proton pump inhibitors, are also reported as risk factors for gastric cancer after *H. pylori* eradication. These studies on the characteristics and predictors of gastric cancer development will become the cornerstone for establishing a novel surveillance program based on the gastric cancer risk stratification specific to *H. pylori*-eradicated patients.

Key words: Gastric cancer; Eradication; Characteristic; *Helicobacter pylori*; Predictor

Core tip: Gastric cancer develops even after successful *Helicobacter pylori* (*H. pylori*) eradication therapy. With the prevalence of eradication therapy, occurrence

rates of gastric cancer detected after eradication are increasing and this is becoming an important clinical issue. We review the characteristics and predictors of primary gastric cancer after *H. pylori* eradication, and discuss the risk stratification of gastric cancer after eradication.

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INTRODUCTION

Gastric cancer is one of the deadliest malignancies, with 1 million cases annually around the world. In the past, the standard curative treatment was surgical resection with lymph node dissection, as the disease was usually diagnosed in the advanced stages in symptomatic subjects. To improve the prognosis of gastric cancer, diagnostic instruments and techniques were developed in Japan, where the occurrence of, and mortality by, gastric cancer were extremely high. Surveillance by endoscopy is one of the methods that enable early diagnosis of gastric cancer. Not only through diagnosis but also by its therapeutic properties[1] endoscopy has changed the management, and improved the prognosis, of gastric cancer. The discovery of the gastric pathogen, *Helicobacter pylori* (*H. pylori*), which was recognized as a group 1 carcinogen[2], dramatically changed the image of gastric cancer from a cryptogenic devastating disease to an infectious, predictable and preventable one[3].

Warren and Marshall isolated *H. pylori* from gastric tissue with gastritis[4]. Initially the pathogenesis of this bacterium was examined in peptic ulcer patients. Developments of diagnostic procedures and antibiotics for *H. pylori* have improved the treatment of peptic ulcers[5-7]. Further research on this pathogen revealed its pathogenesis in relation to chronic gastritis and gastric cancer as well, with early studies demonstrating that *H. pylori* infection increased the risk for gastric cancer[8,9]. Uemura *et al*[10] performed a cohort study of endoscopic surveillance of gastric cancer and found that all occurrences of gastric cancer in the cohort were in *H. pylori*-infected subjects. From these findings, *H. pylori* infection was incorporated into the previously proposed gastric carcinogenesis process known as Correa's cascade[11-13]. Specifically, *H. pylori* infection initiates sequential histological changes such as non-atrophic gastritis, atrophic gastritis[14-16], intestinal metaplasia[15-18], dysplasia, and intestinal-type gastric cancer[19]. In contrast, such a sequential model was not applicable to diffuse-type gastric cancer, though diffuse-type gastric cancer is also associated with *H. pylori* infection[20-22].

Based on these findings, other studies examined the effect of *H. pylori* eradication on preventing gastric cancer. Fukase *et al*[23] reported that metachronous development of gastric carcinoma was reduced by *H. pylori* eradication after endoscopic resection of early gastric cancer in an open-label multicenter randomized controlled trial. Wong *et al*[24] performed a prospective, randomized, placebo-controlled, population-based study to examine the association of primary gastric cancer and eradication therapy. The incidence of gastric cancer development was similar between the eradication-treatment group and placebo group in this study. However, in the subgroup without precancerous lesions, eradication significantly decreased the development of gastric cancer. Recent systematic reviews and meta-analysis showed reduction of primary and metachronous gastric cancer by *H. pylori* eradication[25-27]. Although the effect of *H. pylori* eradication on the prognosis is not determined yet, it is expected that *H. pylori* eradication and elimination within society will lead to less gastric cancer cases and a reduction of medical costs[28].

These reports on the effect of *H. pylori* eradication for gastric cancer also elucidated another important fact. That is, gastric cancer did develop in *H. pylori*-eradicated patients[29,30], not only in gastric cancer-treated patients, who presumably possess the highest risk, but also in gastric-cancer naïve cases[24,31]. Time from eradication to cancer occurrence varied from several months to more than 10 years[31,32]. Therefore, identification of high-risk subjects, who would benefit from extensive surveillance, is an important clinical problem. Many earlier studies have investigated differences in gastric cancers that developed in *H. pylori*-infected and eradicated patients, to assist the early and accurate diagnosis in eradicated cases. Recent studies, which included a relatively large number of *H. pylori*-eradicated cohorts, enabled analysis of the risk factors of future gastric cancer development.

The purpose of this review article was to summarize the characteristics of gastric cancer that developed after *H. pylori*-eradication therapy, and also to identify the predictors of primary gastric cancer. Many previous studies have examined risk factors for metachronous gastric cancer development, in follow-up or cross-sectional studies of endoscopically removed gastric cancer cases[33-37]. Because these cohorts had already developed gastric cancer, they benefited from multiple, surveillance endoscopy as well as *H. pylori* eradication. There are many review articles on this specific topic[38-40]. However, these patients who once had gastric cancer are high-risk patients anyway, and close follow-up should be required. In contrast, a review article for the risk factors in gastric-cancer naïve cases after *H. pylori* eradication, which would be valuable for stratifying huge numbers of *H. pylori*-eradicated patients according to gastric cancer risk, has not been conducted thus far. The findings of this article will be useful for establishing a proper follow-up strategy for *H.*

Table 1 Endoscopic and histological characteristics of gastric cancer after *Helicobacter pylori* eradication

Ref.	Number of gastric cancer after eradication/during infection	Study design	Case recruitment	Characteristics
Shichijo *et al*[31]	21/NA	Case series	Surveillance	Intestinal type
Maehata *et al*[42]	96/96	Propensity score-matched study	ESD cases	Depressed
Nishizawa *et al*[43]	34/NA	Case series	Surveillance	Depressed, intestinal type, relatively small
Matsuo *et al*[44]	26/78	Case control study	Surveillance	Male, intestinal type, flat-depressed, low MUC2 and Wnt5a
Yamamoto *et al*[45]	18/36	Case control study	Early stage cancer	Smaller, lower Ki-67 index, depression, complete gastric type or gastric predominant mixed type
Horiguchi *et al*[48]	71/115	Case control study	Case series	Non-tumorous epithelium Surface differentiation
Ito *et al*[52]	29/NA	Case series	ESD cases	Normal columnar epithelium
Kitamura *et al*[53]	27/27	Case control study	Endoscopic resection cases	Low-grade atypia on the surface
Hori *et al*[54]	59/152	Case control study	Endoscopic resection cases	Non-neoplastic epithelium, flattening of tumors, muting of the whitish discoloration

NA: Not applicable; ESD: Endoscopic submucosal dissection.

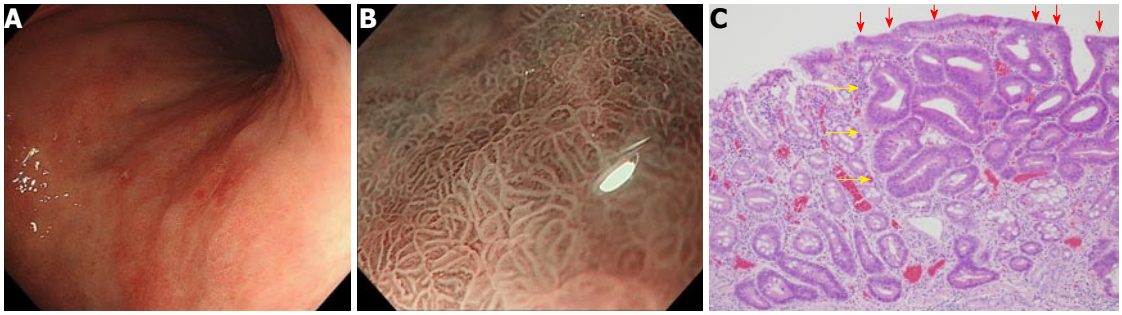

Figure 1 Gastritis-like appearance. A: White light image by conventional endoscopy. Slightly reddish depressed lesion is detected in posterior wall of the upper part of the corpusl; B: A gastritis-like appearance under narrow-band imaging under magnifying endoscopy; C: Well-differentiated tubular adenocarcinoma with low-grade atypia (HE, orig. mag. ×100). Note the non-neoplastic epithelium (arrows) partially covered the surface of the adenocarcinoma (arrowheads).

pylori-eradicated subjects.

ENDOSCOPIC AND HISTOLOGICAL CHARACTERISTICS OF GASTRIC CANCER AFTER *H.PYLORI* ERADICATION

Endoscopic features

Many studies have examined the endoscopic findings of primary gastric cancer after *H. pylori* eradication. Most of these studies were derived from early gastric cancer cases who underwent endoscopic treatment and, therefore, potentially include selection bias.

Depressed lesion: One of the notable endoscopic features of gastric cancer after *H. pylori* eradication is its depressed appearance. Kamada *et al.* reported that among 20 gastric cancer cases discovered in *H. pylori*-eradicated patients, 90% (18 cases) were ulcer type[41]. In a recent and relatively large propensity score-matching study of endoscopic submucosal dissection cases, 81% (78 of 96) of early gastric cancers from *H. pylori*-eradicated patients were depressed type, a significantly higher proportion than the 53% (51 of 96) in *H. pylori*-positive cases[42]. Many other studies, including case series[43], or case control studies[44,45] also indicated predominance of depressed or ulcer type (0-Ⅱc) gastric cancer in *H. pylori*-eradicated cases (Table 1).

Gastritis like appearance: Another important characteristic of gastric cancer after *H. pylori* eradication is its gastritis-like appearance. This was initially reported by Kobayashi *et al*[46]. A "gastritis-like" appearance under narrow-band imaging with magnifying endoscopy was characterized by uniform papillae and/or tubular pits with a whitish border, regular or faint microvessels, and unclear demarcation, resembling the adjacent noncancerous mucosa (Figure 1). They examined retrospectively, differentiated-type early gastric cancer of 50 lesions after eradication, and 50 lesions without eradication. A "gastritis-like" appearance was more frequent for the eradication group (22/50) than the control group (2/50), and the "gastritis-like" appearance correlated with histological surface differentiation[46]. They also reported that the development of "gastritis-like" appearance was associated with less endoscopic atrophy[47]. These phenotypic characteristics or changes make the diagnosis of gastric cancer after *H. pylori* eradication difficult. In fact, it is reported that utilization of chromoendoscopy did not improve the diagnostic

reliability of gastric cancer after *H. pylori* eradication[48]. These endoscopic characteristics of gastric cancer after eradication were associated with histological features that have been termed "non-neoplastic epithelium" (Discussed in the following chapter).

Other: Smaller tumor size is also reported as a characteristic of gastric cancer in *H. pylori*-eradicated cases. Yamamoto *et al*[45] reported that the average diameter of gastric cancer detected after successful eradication was smaller than that in non-eradicated, age, sex, and cancer-depth matched controls. However the control group did not undergo the routine follow-up examination that was performed in the eradicated group. Another propensity-matched study indicated similar tumor size in *H. pylori*-eradicated and infected patients[42].

Histology

Other characteristics of gastric cancer detected after *H. pylori* eradication by histological assessment.

Intestinal type: We have previously conducted a cohort study of 573 *H. pylori*-eradicated cases. During the 6.2 ± 4.8 years of the observation period, we found 21 cases of primary gastric cancer in these *H. pylori*-eradicated patients[31]. Among the 21 tumors, 20 (95%) were intestinal-type gastric cancer, while only one was diffuse type. We did not compare those cancers with non-eradicated cases; however, before eradication therapy, the numbers of intestinal and diffuse-type gastric cancers were roughly even[49]. We speculate that *Helicobacter* eradication could lead to the dominance of intestinal-type gastric cancer. This intestinal-type dominance (*i.e.*, diffuse-type reduction) was also reported in other studies[35,50,51]. However, several studies did not show differences in histological type between *H. pylori*-eradicated and non-eradicated cases[42,45]. These studies analyzed only endoscopic treatment cases, which inevitably exclude diffuse-type cancers. This reduction of diffuse-type gastric cancer by *H. pylori* eradication will be clarified in future large-scale analysis.

Non-neoplastic epithelium: This histological characteristic was initially described in a study that evaluated the histological change of gastric tumors after *Helicobacter pylori* eradication[52]. They named non-neoplastic epithelium which often appeared on the surface of gastric cancer after eradication as epithelium with low-grade atypia (ELA). ELA was observed in 22 out of 27 gastric cancer cases detected after successful eradication where gastric-type mucin was frequently expressed[53]. Hori *et al.* compared 59 tumors detected after eradication and 152 detected while infected, and showed that the histological length ratio of non-neoplastic epithelium to the tumor was 8% for the eradicated group, and 0% for the infected group. The extension of non-neoplastic epithelium has been reported in several other studies (Table 1)[48,54].

Other: One study evaluated Ki67 staining, which is a molecular indicator of cell proliferation. The Ki67 index was lower in the eradicated group than in the non-eradicated group. Immunohistochemical phenotyping revealed that gastric cancer after eradication was more often gastric-predominant type[45]. Another study investigated mucus patterns and Wnt5a expression in gastric cancer specimens derived from *H. pylori*-eradicated and infected patients. The result showed MUC2 and Wnt5a expressions were significantly lower in gastric cancers from *H. pylori*-eradicated patients[44].

PREDICTORS OF PRIMARY GASTRIC CANCER AFTER *H. PYLORI* ERADICATION

In this section, we summarize risk factors for gastric cancer development after *H. pylori* eradication. There are several cohort studies[31,32,55-58] and case-control studies[59-63] on this topic (Table 2). These studies have examined patients' characteristics, endoscopic features, and histological findings associated with gastric cancer after *H. pylori* eradication.

Endoscopic gastric atrophy

The classification of endoscopic atrophy was first described by Kimura and Takemoto in 1969 to discriminate the histological border between the pyloric and fundic glands[64]. They found a close association between this boundary and gastritis. Later, Uemura et al. showed, in their important report, which indicated the critical involvement of *H. pylori* in gastric carcinogenesis, that severe endoscopic atrophy was a risk factor for primary gastric cancer development in *H. pylori*-infected cases[10].

As for patients in whom *H. pylori* had been eradicated, Take et al. investigated risk factors associated with gastric cancer development in 1674 *H. pylori*-eradicated peptic ulcer patients[32]. In their mean 5.6-year surveillance endoscopy program following *H. pylori* eradication, they found 28 cases of gastric cancer in patients with a mean age of 51 years. Patients with severe endoscopic gastric atrophy, which they defined as Kimura-Takemoto classification O2 and O3, before eradication had increased risk for gastric cancer (0.62% per year), compared to patients with mild (C1 and C2) and moderate (C3 and O1) atrophy (0.04% and 0.28% per year, respectively).

We also examined endoscopic atrophy for the prediction of gastric cancer in the above-mentioned study[31]. Multivariate analysis revealed that histologic intestinal metaplasia and severe endoscopic atrophy are independent risks for gastric cancer development. In our study, patients with O2 or O3 atrophy at eradication had 9.3-fold risk for developing gastric cancer compared to patients with no or mild atrophy (C0-C2) in multivariate analysis. Many other cohort studies[55,56] and case-control studies[59,60] showed similar results, that severe endoscopic atrophy is associated with gastric cancer development in *H. pylori*-eradicated patients (Table 2).

Table 2 Risk factor of gastric cancer development after *Helicobacter pylori* eradication

Ref.	Subject number	Study design	Diagnosis	Age	Follow-up period (yr)	Number of cancer	Risk factor
Shichijo *et al*[31]	573	Cohort study	CG/DU/GU	58	6.4	21	Endoscopic severe atrophy Histologic intestinal metaplasia
Take *et al*[32]	1674	Cohort study	GU/DU	51	5.6	28	Endoscopic severe atrophy
Toyoshima *et al*[55]	1232	Cohort study	CG/DU/GU	54	2.5	15	Endoscopic severe atrophy
Sakitani *et al*[56]	965	Cohort study	CG/DU/GU	63	4.5	21	Endoscopic severe atrophy
Cheung *et al*[57]	63397	Cohort study	*Helicobacter pylori* infection (GU 2%, DU 3%)	55	7.6	153	Proton pump inhibitor
Takata *et al*[58]	101	Cohort study	CG/GU/GC	56	5.3	8	Age
Kodama *et al*[59]	2355	Matched control study	CG/DU/GU/GC	63	4.1	21	Endoscopic severe atrophy OLGA staging Histologic atrophy at the antrum Histologic inflammation at the corpus Histologic intestinal metaplasia at the corpus
Sugimoto *et al*[60]	1200	Cross- sectional study	NA	70	4.6	79	Endoscopic severe atrophy
Haneda *et al*[62]	261	Cross sectional study	CG/DU/GU/GC/ MALToma/hyperplastic polyp	57	NA	47	Pepsinogen Ⅰ / Ⅱ ratio ⩽ 4.5
Maeda *et al*[63]	177	Cross sectional study	NA	NA	NA	94	Epigenetic marker

GU: Gastric ulcer; DU: Duodenal ulcer; CG: Chronic gastritis; GC: Gastric cancer; NA: Not available.

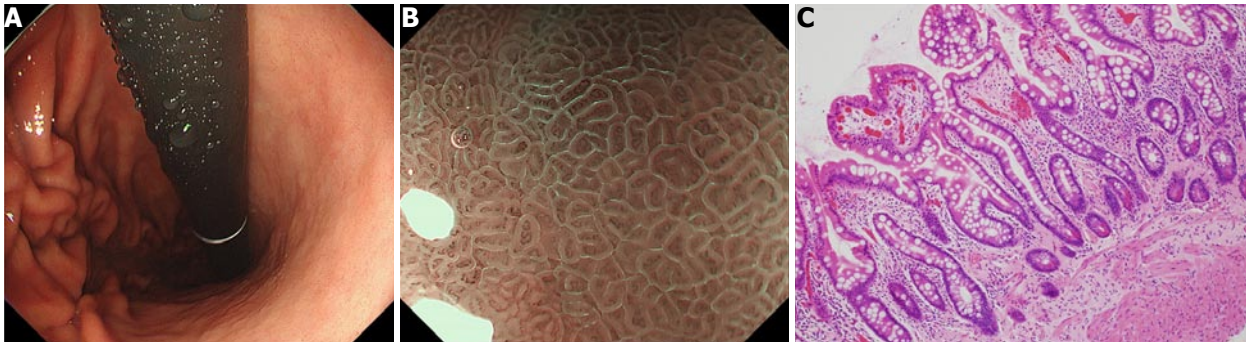

Figure 2 Intestinal metaplasia in the corpus. A: Endoscopic image of the intestinal metaplasia in the corpus. Greyish-white, slightly opalescent, flat, elevated lesions of various sizes; B: Narrow-band imaging under a magnifying endoscopy image, light blue crest appears as blue-white lines visible on the epithelial surface[75]; C: Microscopic picture of a biopsy specimen with intestinal metaplasia (HE, orig. mag. ×100).

Histological intestinal metaplasia

Another well-characterized gastric cancer risk is histological intestinal metaplasia, evaluated at the time of eradication therapy (Figure 2). It has been suggested that intestinal metaplasia precedes gastric cancer development, especially for intestinal-type cancer[11,13]. There have been debates on whether this histological change is a precancerous or a paracancerous lesion, which has not yet been completely clarified. Nonetheless, several observational studies have indicated that the presence of intestinal metaplasia in the background gastric tissue indicates a higher risk for accompanying or developing gastric cancer[10,17,18,65,66].

As described above, we have analyzed 573 endoscopy-based surveillance cases after *H. pylori* eradiation, in which 21 cases of gastric cancer were observed[31]. Before eradication, participants had been evaluated for the presence of intestinal metaplasia[17,18] and neutrophil infiltration using antral and corpus biopsies, and the degree of endoscopic atrophy. We classified patients into three groups according to the histological metaplasia distribution[17,18]. Compared to the group with no intestinal metaplasia, patients with metaplasia limited to antrum had a 4.5-fold increased risk, and patients with metaplasia in corpus had a 7.6-fold increased risk in univariate analysis. Multivariate analysis revealed that the presence of histologic intestinal metaplasia was an independent risk for gastric cancer development. To the best of our knowledge this is the first report that shows intestinal metaplasia as the predictor of future gastric cancer after *H. pylori* eradication.

Kodama *et al*[59] performed cross-sectional and case-control analyses of an *H. pylori*-eradicated cohort and reported that the histological intestinal metaplasia score in the corpus was significantly higher in gastric cancer cases than that in age- and sex- matched non-cancer controls. This study also evaluated the intestinal metaplasia score in the antrum, which did not show a statistical difference between the two groups. Taken together, the presence of intestinal metaplasia, especially in the corpus, might indicate a higher risk for developing gastric cancer.

Long-term use of proton pump inhibitors

Cheung *et al*[57] recently reported, based on a territory-wide health database of Hong Kong, that long term use of proton pump inhibitors (PPI) was associated with an increased gastric cancer risk in subjects after *H. pylori*-eradication therapy. Among 63397 eligible patients who received clarithromycin-based triple therapy between 2003 and 2012, 153 cases of gastric cancer developed before 2015. The risk increased with duration of PPI use (Hazard ratio 5.0, 6.7, and 8.3 for ⩾ 1, 2, and 3 years, respectively). Many researchers quickly responded to this topic, and both affirming[67] and contradicting reports[68,69] followed this paper. Interestingly, another population-based study in Sweden also indicated the increased risk of gastric cancer (regardless of *H. pylori* infection or eradication) in maintenance therapy with PPI[70]. Contrary to the former study on *H. pylori*-eradicated cases[57], this study did not find that the risk increased with therapeutic duration. Therefore, this topic still requires more study before a consensus can be reached, but clinicians should take PPI use into account for future studies of gastric cancer risk assessment.

Other

Age factor has also been reported in many studies. Most studies showed an older age at eradication is associated with an increased risk of developing cancer in univariate analysis[31,55,59]. However, age is also closely associated with other gastritis-related phenotypes, which often lead to less value in carcinogenesis under multivariate analysis. For example, a cohort study examined 101 histologically diagnosed corpus atrophic gastritis patients who underwent successful eradication therapy[58]. This study found eight gastric cancer cases (all intestinal type) during a mean follow-up period of 5.3 year, and the patients' characteristics and serum biochemistry data were compared in the groups with and without cancer. Out of age, sex, the disease indicated for eradication (gastritis or gastric ulcer), prior gastric cancer, pepsinogen value, and gastrin value, only age (64 years *vs* 55 years) was statistically different between groups. However, lack of multivariate analysis or other important confounding factors, such as endoscopic atrophy or histological metaplasia, might have led to an immature conclusion in this study.

Endoscopic diagnosis associated with *H. pylori* infection has been examined as a risk or protective factor for gastric cancer development. In a *H. pylori*-persistent infection cohort, reduced risk for gastric cancer development was found specifically in duodenal ulcer patients (0 out of 275 for duodenal ulcer *vs* 36 out of 971 for other diseases)[10]. As for patients after eradication therapy, Kamada *et al*[41] reported that no gastric cancer developed in 654 duodenal ulcer patients, while 12 of 575 (2.1%), two of 453 (0.4%), and six of 105 (5.7%) cases were reported in gastric ulcer, atrophic gastritis and endoscopic resection for early gastric cancer patients, respectively. Kodama *et al*[59]. also reported only three gastric cancer cases, developed from 655 patients with duodenal ulcers (0.5%), while 10 of 902 (1.1%), 14 of 593 (2.4%), and 3 of 51 (5.9%) cases developed from patients with chronic gastritis, gastric ulcers, and gastric cancer, respectively. These reports indicate duodenal ulcer patients who received eradication therapy have less risk for future gastric cancer occurrence than do patients who have undergone eradication for other *H. pylori*-related diseases.

Pepsinogen (PG) methods are clinically used for the gastric-cancer screening program in Japan. As low PGI levels and low PGI/Ⅱ ratios are correlated with mucosal atrophy, the efficacy of this screening method for identifying high-risk subjects of gastric cancer has been reported in multiple cohort studies[71,72]. However PG values and ratios change after *H. pylori* eradication[73], and the usefulness of the PG method in *H. pylori*-eradicated patients was not evident. Haneda *et al*[62]. examined PG levels in post-eradication cases with and without gastric cancer, and found that the optimal cut-off value for the PGI/Ⅱ ratio was 4.5 (instead of the usual 3.0). The usefulness of this cut-off value in clinical practice needs to be confirmed in a cohort or a prospective study.

Finally, molecular indicators of gastric cancer risk have been investigated intensively. Recent research, focused on epigenetic markers, has revealed completely new types of gastric cancer risk predictors. In a case-control study consisting of eight cases without infection, 75 atrophic gastritis post-eradication cases and 94 gastric cancer post-eradication cases, nine candidate epigenetic markers, which showed elevated methylation levels in cancer cases, were isolated[63]. These new markers are now being evaluated in a prospective cohort study, which will elucidate the clinical usefulness of these molecular approaches in the near future.

PERSPECTIVES

Here we have reviewed characteristics and predictors of gastric cancer after *H. pylori* eradication. Knowledge of endoscopic characteristics, such as depressed and gastritis-like appearance, and an understanding of the histological non-neoplastic epithelium, will be helpful in detecting gastric cancer while screening subjects after eradication therapy. Reportedly, the tumors detected after *H. pylori* eradication seemed to be less proliferative and more gastric phenotype. This might be associated with a differentiation program by adult-tissue stem cells, and the mechanism of these molecular changes and the effect of *H. pylori* eradication will be an interesting research project. As for predictors, severe endoscopic atrophy, histologic intestinal metaplasia before eradication, and PPI use are reportedly risk factors for gastric cancer development after eradication. Cases with these risk factors should be carefully followed up by endoscopy, with special attention paid to the aforementioned characteristic endoscopic findings.

So far, most of the risk factors were evaluated before the eradication, which is helpful for identifying high-risk patients early so they can be invited into a surveillance program. However, risk stratification according to findings after eradication, not those before eradication, might be more practical, because information prior to eradication is not always available. For example, we proposed that histological intestinal metaplasia is an important risk factor for future gastric cancer development, but most of the *H. pylori*-eradicated subjects in the community did not receive a histological evaluation prior to eradication. If there is little change in the metaplasia after eradication, then assessment of histology after eradication may be used as a substitute, but this would need to be evaluated by independent studies. Risk stratification by pepsinogen levels after eradication has been reported[62], but further validation studies are necessary. Some researchers have focused on endoscopic changes after *H. pylori* eradication that was accompanied with cancer[74]. Map-like redness after eradication, which corresponds to intestinal metaplasia histologically, could be a predictor for metachronous gastric cancer. Importantly, recent retrospective epigenetic research used gastric samples collected from post-eradication cases, which is ideally applicable to all subjects[63]. Therefore the result of the ongoing prospective study is highly anticipated.

Eradication therapy is relatively new, and current studies are mostly limited to elder patients over 50 years old. Therefore, further long-term follow-up studies, over several decades, or a study of the young population should be required to form a consensus for an adequate surveillance program. Based on the results reviewed in this paper, it is safe to propose annual endoscopic surveillance for high-risk *H. pylori*-eradicated patients, such as those with severe endoscopic atrophic gastritis (O2 or O3) or histological intestinal metaplasia before eradication. Patients who require PPI treatment for any reason after eradication should also have an annual checkup for both gastric cancer surveillance and for the conditions requiring PPI. However, for other relatively low-risk eradicated patients, such as subjects with mild atrophy or no metaplasia, little evidence exists to propose a proper surveillance program. As these relatively low risk patients consist of the majority of *H. pylori*-eradicated cases, studies targeting these subjects will definitely be required. New studies, new modalities, and new concepts will lead to the establishment of a primary gastric cancer surveillance program suitable for all *H. pylori*-eradicated cases according to their cancer risk stratification.

CONCLUSION

In this review article, we have summarized the previous studies on the characteristics and predictors of gastric cancer which developed after successful *H. pylori* eradication. Gastric cancer surveillance program after *H. pylori* eradication according to risk stratification needs to be established in future.

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ORIGINAL ARTICLE

Basic Study

Effects of hepatitis E virus infection on interferon production *via* ISG15

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Abstract

AIM

To assess the effects of hepatitis E virus (HEV) on the production of type Ⅰ interferons (IFNs) and determine the underlying mechanisms.

METHODS

We measured the production of interferon (IFN)-alpha and -beta (-α/β) in genotype 3 HEV-infected C3A cells at different time points (0, 8, 12, 24, 48, 72 and 120 h) by enzyme-linked immunosorbent assay (ELISA). The expression levels of IFN-stimulated gene (ISG)15 in HEV-infected C3A cells at different time points were tested by western blotting. The plasmid-expressing open reading frame 3 (ORF3) or control plasmids (green fluorescent protein-expressing) were transfected into C3A cells, and the levels of IFN-α/β and ISG15 were evaluated, respectively. Furthermore, the plasmid-expressing ISG15 or small interfering RNA-inhibiting ISG15 was transfected into infected C3A cells. Then, the production of IFN-α/β was also measured by ELISA.

RESULTS
We showed that genotype 3 HEV could enhance the production of IFN-α/β and induce elevation of ISG15 in C3A cells. HEV ORF3 protein could enhance the production of IFN-α/β and the expression of ISG15. Additionally, ISG15 silencing enhanced the production of IFN-α/β. Overexpression of ISG15 resulted in the reduction of IFN-α/β.

CONCLUSION
HEV may promote production of IFN-α/β and expression of ISG15 *via* ORF3 in the early stages, and increased ISG15 subsequently inhibited the production of IFN-α/β.

Key words: Open reading frame 3; Interferon-stimulated gene 15; Interferon-alpha; Hepatitis E virus; Interferon-beta

Core tip: To date, few studies have investigated the role of ISG15 in hepatitis E virus (HEV) infection and the impact of ISG15 on interferons (IFN) production. This study showed that HEV could inhibit the level of type Ⅰ IFN through regulating the expression of IFN-stimulated gene (ISG)15, which may attenuate the IFN response, allowing for successful infection of their hosts. The present study enhances the understanding of the interaction between ISG15 and HEV in the host innate immune response, which may provide useful insight for the development of new antiviral drugs and antiviral strategies.

Wang M, Huang Y, He M, Peng WJ, Tian DY. Effects of hepatitis E virus infection on interferon production *via* ISG15. *World J Gastroenterol* 2018; 24(20): 2173-2180 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i20/2173.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i20.2173

INTRODUCTION

Hepatitis E virus (HEV) infection is one of the most common causes of acute hepatitis or sporadic acute hepatitis in many parts of the world[1]. At least 20 million HEV infections occur annually, which are mostly self-limiting and have different clinical manifestations, such as asymptomatic, acute liver failure and rare chronicity[2,3]. In some cases, HEV infections can cause up to 30% mortality in pregnant women[4] and may result in chronic hepatitis in immunocompromised individuals, such as those receiving organ transplants or chemotherapy, and individuals infected with human immunodeficiency virus[5-7]. Currently, at least four genotypes exist among HEV strains, of which HEV genotypes 1 and 2 are obligate human pathogens and HEV genotypes 3 and 4 are mostly zoonotic[8]. Recently, a study found that a genotype 3 HEV isolated from a chronically infected patient could be adapted to grow in human C3A hepatoma cells[9].

HEV has a 7.2-kb single-stranded, positive-sense RNA genome containing three open reading frames (ORFs)[10]. The ORF1 protein comprises the functional domains of virions. ORF2 is the major structural protein of virions and encodes the viral capsid protein. And, ORF3 protein, a small molecule phosphoprotein, is involved in viral particle secretion[11]. A recent study found that chronic HEV infections were almost exclusively caused by the zoonotic genotype 3 HEV strains[12].

Type Ⅰ interferons (IFNs) are key components of innate immunity and known to be the first-line defense against virus infection. IFN exerts many biological effects, such as antiviral and antitumor activity, and immune regulation by activating hundreds of downstream IFN-stimulated genes (ISGs)[13,14]. One of the most abundantly IFN-induced proteins is ISG15, which is encoded and expressed as a ubiquitin-like protein. ISG15 is covalently attached to target proteins through a C-terminal LRLRGG motif, in a process called ISGylation. ISGylation could alter protein properties directly by addition of ISG15, as well as by reducing the degradation of the target protein by competing with ubiquitin conjugation[15]. Previous studies have shown that ISG15 is critical for control of certain viral and bacterial infections and is linked to the process of budding or egress for many RNA virus families[16-18].

As few studies have focused on the role of ISG15 in HEV infection, we investigated whether HEV infection could regulate the expression of ISG15 and the impact of ISG15 on IFN production during HEV infection in the present study, which is of great significance to expand our understanding of the interaction between ISG15 and HEV pathogenesis.

MATERIALS AND METHODS

Cells and virus

C3A is a derivative of HepG2 cells (hepatoma cells). C3A cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, United States) and cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (Gibco, Thermo Scientific, Waltham, MA, United States), penicillin (100 U/mL) and streptomycin (100 μg/mL) at 37 ℃ with 5% CO_2. The genotype 3 Kernow-C1 HEV infectious cDNA clone was a kind gift from the National Institute of Diagnostics and Vaccine Development in Infectious Diseases (NIDVD, Xiamen, China). The ATG start codon of ORF3 in Kernow-C1 HEV was altered to GTG to construct an HEV ORF3 mutant, which led to the loss of ORF3 protein expression.

Reagents and plasmids

Anti-ISG15 antibody was purchased from Signalway Antibody (42509, 1:1000 dilution; Pearland, TX, United States). AntiHEV ORF3-specific antibody was purchased from Abbiotec (250688, 1:1000; San Diego, CA, United States). Primary antibody against β-actin (#BM0627, 1:400 dilution) was purchased from Boster (Wuhan, China). We constructed a plasmid-expressing

Table 1 Primers used in this study

Name	Primer	Sequence
β-actin	Forward	5′- AGCGAGCATCCCCCAAAGTT -3′
	Reverse	5′- GGGCACGAAGGCTCATCATT -3′
ISG15	Forward	5′- GCGCAGATCACCCAGAAGAT -3′
	Reverse	5′- GTTCGTCGCATTTGTCCACC -3′
siRNA1	Forward	5′- UGUCGGUGUCAGAGCUGAATT -3′
	Reverse	5′- UUCAGCUCUGACACCGACATT -3′
siRNA2	Forward	5′- GAUGCUGGCGGGCAACGAATT -3′
	Reverse	5′- UUCGUUGCCCGCCAGCAUCTT -3′
siRNA3	Forward	5′- UGAGCACCGUGUUCAUGAATT -3′
	Reverse	5′- UUCAUGAACACGGUGCUCATT -3′
Negative control	Forward	5′- UUCUCCGAACGUGUCACGUTT -3′
	Reverse	5′- ACGUGACACGUUCGGAGAATT -3′

ORF3 (ORF3) as well as a plasmid-expressing ISG15 (ISG15). ORF3 and ISG15 fragments were cloned into the pEGFP-N1 vector (green fluorescent protein (GFP)-expressing, GenBank accession #55762) by digesting with *Xho* Ⅰ and *Hind* Ⅲ to construct the pORF3-EGFP plasmid (ORF3) and pISG15-EGFP plasmid (ISG15). All the clones were confirmed by restriction digestion and DNA sequencing.

HEV infection

Th cDNA clones were linearized with *Bgl* Ⅱ, and the genomic RNA was transcribed using the MEGAscript Kit (Ambion, Thermo Scientific). C3A cells cultured in a six-well plate were transfected with capped RNA transcripts from Kernow-C1 HEV infectious cDNA clone using DMRIE-C reagent (Invitrogen, Grand Island, NY, United States) following the manufacturer's protocol. The ratio of RNA to DMRIE-C was 2 μg:8 μL. After 6 h, the medium in the six-well plate was removed and replaced with serum-free medium. The cells and cell culture supernatants were harvested at different time points (0, 8, 12, 24, 48, 72 and 120 h) and used in experiments.

Cell transfection

C3A cells were plated in six-well microplates and transfected with a plasmid-expressing ORF3-EGFP (ORF3) or the HEV mutant, leading to the loss of ORF3 protein expression (ΔORF3) using Lipofectamine TM 3000 Transfection Reagent (Invitrogen, San Diego, CA, United States) for 48 h. Then, the cells and cell culture supernatants were harvested at different time points (0, 8, 12, 24, 48, 72 and 120 h) and used in experiments.

Small interfering RNA-mediated knockdown

The small interfering RNAs (siRNAs) targeted against ISG15 were purchased from Santa Cruz Biotechnologies (Dallas, TX, United States). C3A cells were transiently transfected with 20 nmol/L of siRNA using the DMRIE-C reagent (Invitrogen) according to the manufacturer's protocol. Primers are shown in Table 1.

Enzyme-linked immunosorbent assay

The concentrations of IFN-alpha and -beta (-α/β) were evaluated in cell culture supernatants using a human IFN-α/β ELISA kit (Joyee Biotechnics, Shanghai, China). All samples were tested in triplicate.

Western blot analysis

Cells were lysed in RIPA buffer (Beyotime Biotechnology, Shanghai, China), and the protein concentration was determined using an enhanced BCA protein assay kit (Beyotime Biotechnology). Ten micrograms of each protein sample were separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and subsequently transferred to polyvinylidene difluoride membranes. Samples were then incubated with specific primary antibodies overnight, following incubation with an antimouse secondary antibody solution for 2 h at room temperature. The blotted membranes were exposed to film, and the protein bands were visualized by a scanner system.

Real-time reverse transcriptase-polymerase chain reaction

Total RNA from C3A cells was isolated using Trizol (Thermo Scientific) and reverse-transcribed into cDNA using the *Taq*Man Reverse Transcription Reagents (Thermo Scientific). The mRNA level of ISG15 was quantified by real-time PCR using SYBR master mix (Thermo Scientific). The PCR amplification conditions included 1 cycle of 95 ℃ for 2 min, and 40 cycles of 95 ℃ for 10 s, 60 ℃ for 20 s, and 72 ℃ for 30 s; the Roche Light Cycler 480 Ⅱ Real-Time PCR System was used. Threshold cycle (CT) values were determined by RT-PCR and normalized by the housekeeping gene β-actin. The mRNA abundance was calculated using the $2^{-\Delta\Delta Ct}$ method and expressed as fold-change. Primers are shown in Table 1.

Statistical analysis

All the data are presented as mean ± SD. Statistical differences between the samples and the controls were assessed by the Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

HEV infection enhances the production of IFN-α/β and induces elevation of ISG15 protein in C3A cells

In this study, C3A cells were transfected with HEV RNA.

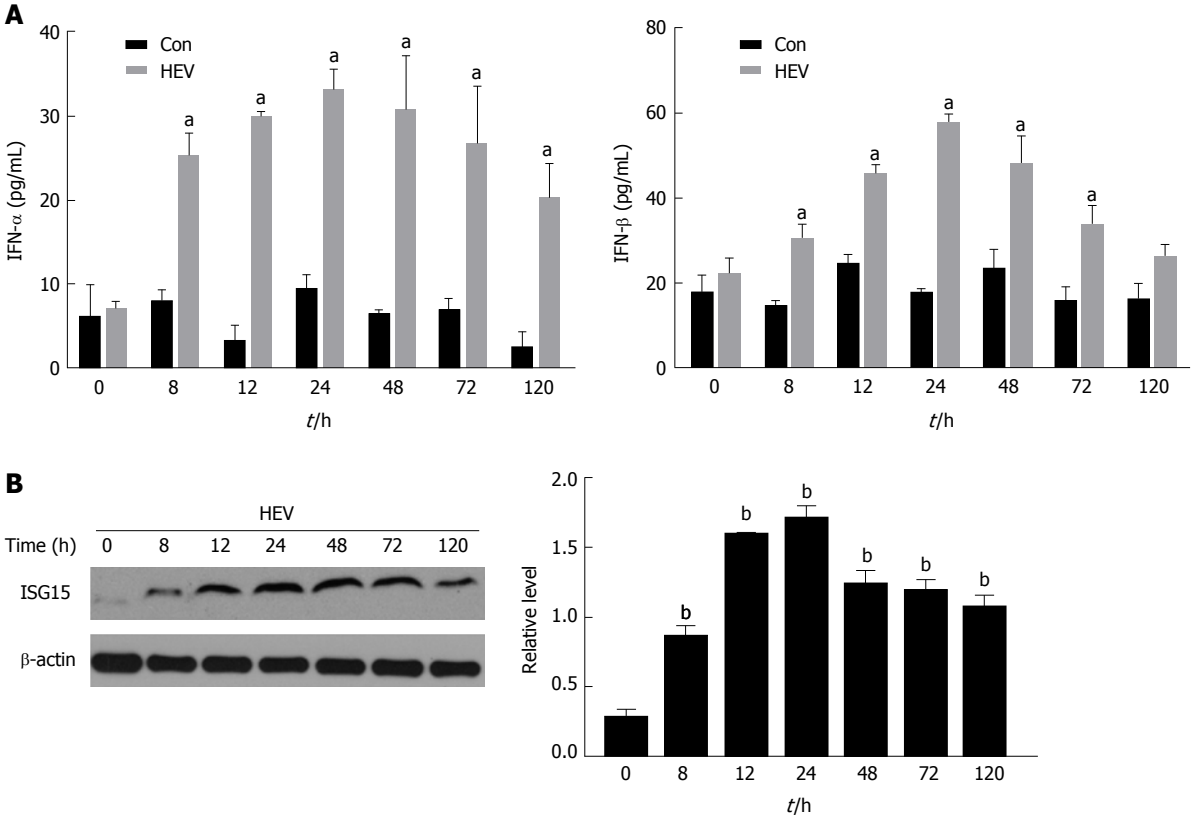

Figure 1 Hepatitis E virus infection enhances the production of Interferon-α and -β and induces elevation of Interferon-stimulated gene 15 protein in C3A cells. A: The concentrations of IFN-α and β were evaluated in cell culture supernatants at different time points (0, 8, 12, 24, 48, 72 and 120 h) by enzyme-linked immunosorbent assay; B: The expression of ISG15 protein replication was detected at different time points (0, 8, 12, 24, 48, 72 and 120 h) by western blotting. The data are expressed as mean ± standard error of the mean. $^{a}P < 0.05$, $^{b}P < 0.01$. HEV: Hepatitis E virus; IFN: Interferon; ISG: IFN-stimulated gene.

To determine whether HEV infection could affect the production of type Ⅰ IFN, the concentrations of IFN-α/β were evaluated in cell culture supernatants at different time points (0, 8, 12, 24, 48, 72 and 120 h) by ELISA. The levels of IFN-α/β in the HEV infection group were significantly increased at each time point ($P < 0.05$), and the levels of IFN-α/β reached the maximum at 24 h, while no significant changes were observed in the control group (Figure 1A).

Next, we identified whether HEV could induce ISG15. ISG15 protein expression was detected at different time points by western blotting. It showed HEV enhanced the expression of ISG15 ($P < 0.01$) and ISG15 protein expression reach the maximum at 24 h as compared to the control group (Figure1B).

HEV ORF3 protein enhances the production of IFN-α/β and the expression of ISG15 protein in C3A cells

To investigate how HEV enhances the production of IFN-α/β and induces elevation of ISG15, we constructed a plasmid-expressing ORF3-EGFP (ORF3) and the HEV mutant leading to the loss of ORF3 protein expression (ΔORF3). C3A cells were transfected with ORF3 or ΔORF3 respectively, and the production of IFN-α/β and the expression of ISG15 protein were detected. The production of IFN-α/β was significantly increased at each time point in the group transfected with ORF3 plasmid (ORF3 group) compared to the ORF3-lacking group (ΔORF3 group) ($P < 0.05$). In addition, the concentrations of IFN-α/β increased the maximum at 24 h (Figure 2A). The ORF3 group could induce higher ISG15 protein expression than ΔORF3 group ($P < 0.01$). ISG15 protein was poorly expressed in the early stage, from 0 h to 12 h, and significantly increased at 24 h, reaching the maximum at 72 h (Figure 2B).

ISG15 silencing enhances the production of IFN-α/β in C3A cells

To investigate the levels of IFN-α/β when ISG15 was inhibited, we first constructed three siRNAs that could inhibit the expression of ISG15 and then transfected them into C3A cells, respectively. Then, the mRNA and protein levels of ISG15 were detected to determine which siRNA had the best inhibitory effect (Figure 3A and B). The siRNA2 was found to have the best inhibitory effect on ISG15. Next, the siRNA2 was transfected into C3A cells that had been pretreated with HEV RNA. The expression of IFN-α was significantly increased in the HEV-infected C3A cells transfected with siISG15 compared to the HEV-infected cells ($P < 0.05$). Consistently, the expression of IFN-β showed the same trend ($P < 0.01$) (Figure 3C).

Overexpression of ISG15 results in the reduction of IFN-α/β in C3A cells

To investigate the effect of ISG15 overexpression on IFN-α/β, the plasmid -expressing ISG15 was constructed and transfected into C3A cells. The level of ISG15 was

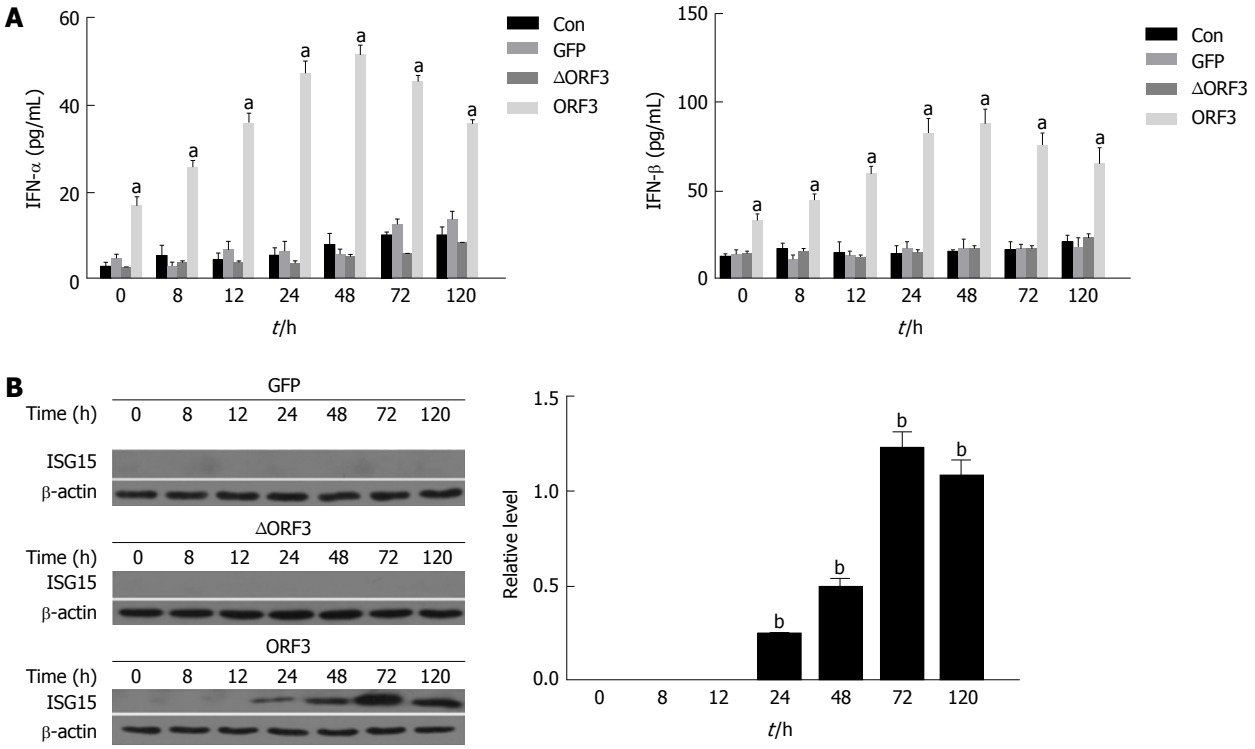

Figure 2 Hepatitis E virus open reading frame 3 protein enhances the production of Interferon-α and β and the expression of Interferon-stimulated gene 15 protein in C3A cells. C3A cells were transfected with 2 μg of either pORF3-EGFP (ORF3) or pEGFP-N1 empty vector (GFP), and the HEV mutant, leading to the loss of ORF3 protein expression (ΔORF3) for 48 h, untreated cells were used as the control (Con). Then, the cells and cell culture supernatants were harvested at different time points (0, 8, 12, 24, 48, 72 and 120 h). A: The concentrations of IFN-α and β were evaluated in cell culture supernatants at different time points (0, 8, 12, 24, 48, 72, 120 h) by enzyme-linked immunosorbent assay; B: The expression of ISG15 protein was detected at different time points (0, 8, 12, 24, 48, 72 and 120 h) by western blotting. The data are expressed as mean ± standard error of the mean. $^{a}P < 0.05$, $^{b}P < 0.01$. GFP: Green fluorescent protein; HEV: Hepatitis E virus; IFN: Interferon; ISG: IFN-stimulated gene; ORF: Open reading frame.

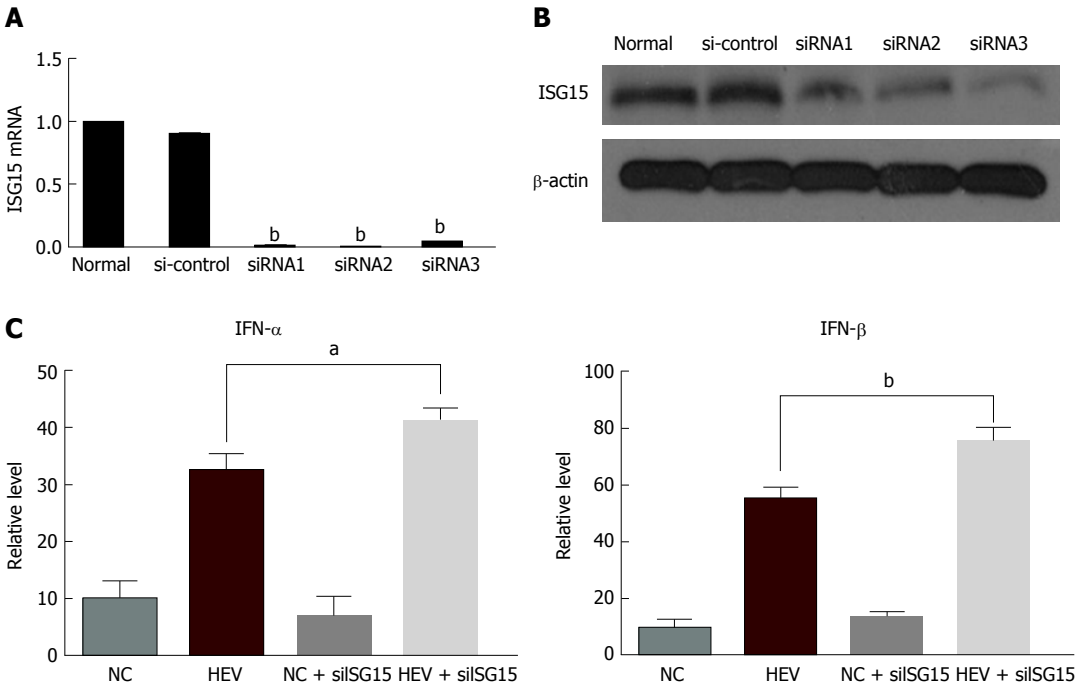

Figure 3 Interferon-stimulated gene 15 silencing enhances the production of Interferon-α/β in C3A cells. Three siRNA inhibiting ISG15 (siISG15) and control-siRNA (si-control) were constructed, and C3A cells were cotransfected with 20 nmol/L ISG15-targeted siRNA (siISG15) or control-siRNA (si-control) along with HEV RNA. A: The mRNA level of ISG15 was estimated by reverse transcription-PCR; B: The protein level of ISG15 was estimated by western blotting. Next, the siRNA2 was transfected into C3A cells that had been pretreated with HEV RNA, and the impact of siISG15 on IFN was investigated; C: The level of IFN-α/β was tested by enzyme-linked immunosorbent assay. The data are expressed as mean ± standard error of the mean. $^{a}P < 0.05$, $^{b}P < 0.01$. HEV: Hepatitis E virus; IFN: Interferon; ISG: IFN-stimulated gene; si: Small interfering.

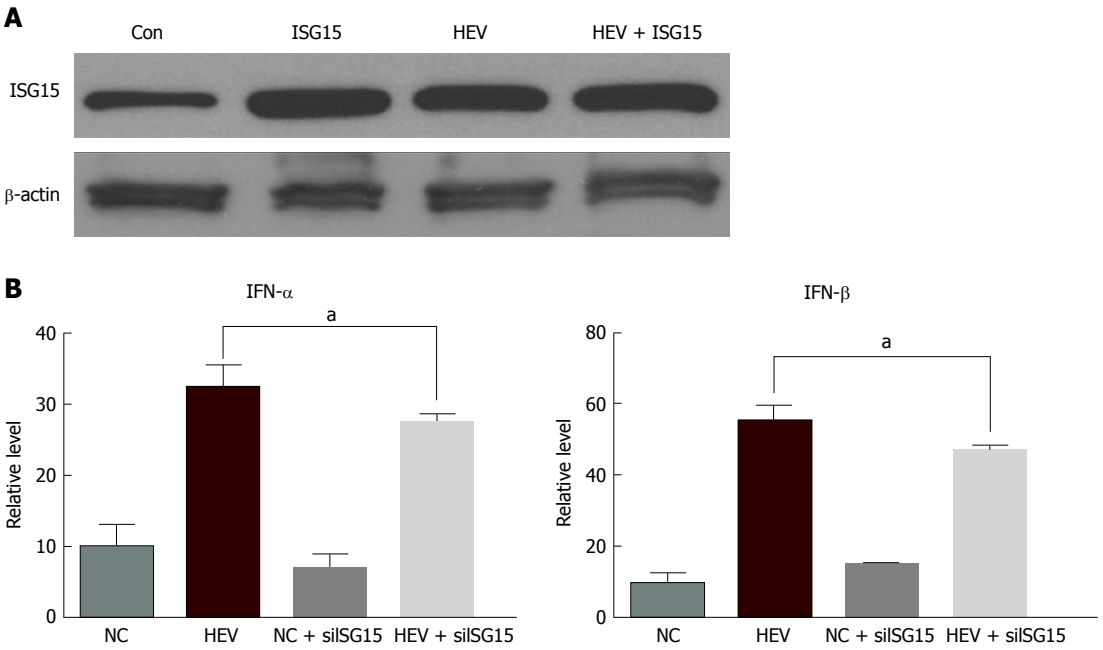

Figure 4 Over-expression of Interferon-stimulated gene 15 results in the reduction of Interferon-α/β in C3A cells. C3A cells were transfected with 2 mg ISG15-EGFP plasmid (ISG15) along with HEV RNA. A: ISG15 protein expression was detected by western blotting; B: The levels of IFN-α/β were detected by enzyme-linked immunosorbent assay. [a]*P* < 0.05. HEV: Hepatitis E virus; IFN: Interferon; ISG: IFN-stimulated gene.

detected by western blotting. The cells transfected with ISG15 plasmid had a considerably higher ISG15 level than the control cells (Figure 4A). Then, the production of IFN-α/β was tested when cells were transfected with ISG15 plasmid and HEV RNA. The level of IFN-α/β was decreased when the cells were treated with HEV and ISG15 plasmid ($P < 0.05$) (Figure 4B), which indicated over-expression of ISG15 may result in reduction of IFN-α/β.

DISCUSSION

The innate immune system is the first line of defense against invading pathogens. Type Ⅰ IFNs, such as IFN-α/β, make up part of the innate immunity system and are critical for innate immunity against viral infection. ISG15, a member of the ISGs induced by IFN, has an important role in the regulation of antiviral immunity. In this study, we demonstrated that HEV infection could enhance the production of IFN-α/β and induce elevation of ISG15 protein. ORF3 protein may be responsible for the enhancement of IFN-α/β and ISG15 by HEV. Furthermore, ISG15 silencing enhanced the production of IFN-α/β in C3A cells. Over-expression of ISG15 resulted in the reduction of IFN-α/β in C3A cells. Collectively, these results indicated that HEV could induce the production of IFN and ISG15, and the increased ISG15 in turn reduced the level of IFN, which revealed a possible correlation between HEV infection and IFN production by regulating the expression of ISG15.

In our study, we first found that HEV infection could enhance the production of IFN-α/β and induce the elevation of ISG15 protein. A previous study demonstrated that most viruses have the ability to regulate the IFN response during infection[19]. When cells are infected by viruses, such as influenza viruses, the activated host innate immune response will lead to secretion of type Ⅰ IFN. Then, the induced IFN exerts its antiviral activity[20]. Over the last decade, numerous studies have reported that ISG15 plays a crucial role against viral infection[21,22]. However, few studies have investigated the effects of HEV on ISG15. In the present study, we found that the expression of ISG15 and the IFN level were simultaneously increased during HEV infection, which indicated that HEV could induce ISG15 expression in C3A cells. Recent studies have revealed that ISG15, as an innate immune protein with broad spectrum antiviral activity, continues to accumulate rapidly when the host is infected by a virus[23], which is consistent with the findings in this research.

HEV consists of three ORFs. Genotype 1 HEV strain could inhibit type Ⅰ IFN induction by ORF1 products[24]. However, the ORF3 products of genotype 1 HEV have been reported to enhance the production of IFN[25]. Because we found that genotype 3 HEV could induce the level of IFN, we next tested the impact of HEV ORF3 on IFN. We found that HEV ORF3 could increase the levels of IFN-α/β, which indicates that HEV ORF3 is responsible for the regulation of IFN. Similarly, we investigated whether HEV ORF3 could affect the expression of ISG15. As we expected, the level of ISG15 was increased by HEV ORF3. Previous studies have shown that ORF3 protein is associated with the egress of HEV[26]. As ISG15 is also associated with the process of egress for many RNA virus families[27], we supposed that HEV ORF3 may regulate the egress of HEV through ISG15, which

requires further experiments to confirm.

Furthermore, the possible correlation between enhanced levels of IFN and ISG15 during HEV infection was investigated. ISG15 silencing enhanced the production of IFN-α/β, and over-expression of ISG15 resulted in the reduction of IFN-α/β in C3A cells, indicating that ISG15 is a negative regulator of IFN. It has been indicated that the absence of intracellular ISG15 in the ISG15-deficient patients prevents the accumulation of USP18, which is a potent negative regulator of IFN-α/β signaling, thus resulting in the enhancement and amplification of IFN-α/β responses and increase of viral resistance in humans[15,28].

In addition, it was found that ISG15 over-expression inhibited induction of IFN-β by HCV infection[29]. Evidence revealing high hepatic ISG15 levels was associated with low antiviral IFN-response during the early phase of antiviral therapy[30], supporting the notion that ISG15 is a negative regulator of the IFN system. However, this raises the question of why the levels of IFN-α/β could be reduced by HEV infection via ISG15. As we know, the IFN production is always enhanced to promote an antiviral state in an infected host, but viruses have evolved many strategies to antagonize the induction of IFNs. As there is little knowledge about how HEV inhibits induction of IFN, the result that the level of IFN could be inhibited by HEV *via* ISG15 will enhance our fundamental knowledge of the mechanisms of induction and evasion of type Ⅰ IFN responses by HEV.

In conclusion, the findings of the present study showed that HEV could inhibit the level of type Ⅰ IFN through regulating the expression of ISG15, which may attenuate the IFN response, allowing for successful infection of the host. The present study enhances the understanding of the interaction between ISG15 and HEV in the host innate immune response, which may provide useful insight for the development of new antiviral drugs and antiviral strategies.

ARTICLE HIGHLIGHTS

Research background

Hepatitis E virus (HEV) infection is one of the most common causes of acute hepatitis or sporadic acute hepatitis in the world. At least 20 million HEV infections occur annually, which may result in chronic hepatitis in immunocompromised individuals. However, the mechanism of HEV pathogenesis remains obscure.

Research motivation

Over the last decade, numerous studies have reported that interferon (IFN)-stimulated gene (ISG)15 plays a crucial role against viral infection. However, few studies have investigated the effects of HEV on ISG15.

Research objectives

In the present study, we investigated whether HEV infection could regulate the expression of ISG15 and the impact of ISG15 on IFN production during HEV infection, which is of great significance to expand our understanding of the interaction between ISG15 and HEV pathogenesis.

Research methods

In this study, C3A cells were first transfected with genotype 3 HEV RNA. The production of IFN-alpha and -beta (IFN-α/β) at different time points (0, 8, 12, 24, 48, 72 and 120 h) were measured by enzyme-linked immunosorbent assay (ELISA). The expression levels of ISG15 in HEV-infected C3A cells at different time points were tested by western blotting. Then, C3A cells were transfected with plasmid-expressing open reading frame 3 (ORF3) or control plasmids, the levels of IFN-α/β and ISG15 was evaluated, respectively. Next, the plasmid-expressing ISG15 or small interfering RNA-inhibiting ISG15 was transfected into infected C3A cells. The production of IFN-α/β was also measured by ELISA.

Research results

In this study, we demonstrated that HEV infection could enhance the production of IFN-α/β and induce elevation of the ISG15 protein. ORF3 protein may be responsible for the enhancement of IFN-α/β and ISG15 by HEV. Furthermore, ISG15 silencing enhanced the production of IFN-α/β in C3A cells. Over-expression of ISG15 resulted in the reduction of IFN-α/β in C3A cells.

Research conclusions

The findings of the present study showed that HEV could inhibit the level of type Ⅰ IFN through regulating the expression of ISG15, a finding which may enhance the understanding of the interaction between ISG15 and HEV in the host innate immune response, and provide useful insight for the development of new antiviral drugs and antiviral strategies.

Research perspectives

The future research will focus on whether HEV ORF3 could regulate the egress of HEV through ISG15.

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ORIGINAL ARTICLE

Retrospective Study

Indoleamine-2,3-dioxygenase 1/cyclooxygenase 2 expression prediction for adverse prognosis in colorectal cancer

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Author contributions: All authors helped to perform the research; Ma WJ performed the manuscript writing, experimental procedures and data analysis; Wang X performed the experiments and data analysis; Yan WT contributed to writing of the manuscript and data analysis; Zhou ZG contributed to collecting data and study design; Pan ZZ contributed to data analysis and study design; Chen G contributed to writing of the manuscript and drafting of the study conception; Zhang RX contributed to study design and data analysis.

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Abstract

AIM

To evaluate indoleamine-2,3-dioxygenase 1/cyclo-oxygenase 2 (IDO1/COX2) expression as an independent

prognostic biomarker for colorectal cancer (CRC) patients.

METHODS

We retrospectively studied the medical records of 95 patients who received surgical resection from August 2008 to January 2010. All patients were randomly assigned to adjuvant treatment with or without celecoxib groups after surgery. We performed standard immunohistochemistry to assess the expression levels of IDO1/COX2 and evaluated the correlation of IDO1/COX2 with clinicopathological factors and overall survival (OS) outcomes.

RESULTS

The expression of nuclear IDO1 was significantly correlated with body mass index (P < 0.001), and IDO1 expression displayed no association with sex, age, tumor differentiation, T stage, N stage, carcinoembryonic antigen, cancer antigen 19-9, CD3+ and CD8+ tumor infiltrating lymphocytes, and COX2. In univariate analysis, we found that nuclear IDO1 (P = 0.039), nuclear/cytoplasmic IDO1 [hazard ratio (HR) = 2.044, 95% confidence interval (CI): 0.871-4.798, P = 0.039], nuclear IDO1/COX2 (HR = 3.048, 95%CI: 0.868-10.7, P = 0.0049) and cytoplasmic IDO1/COX2 (HR = 2.109, 95%CI: 0.976-4.558, P = 0.022) all yielded significantly poor OS outcomes. Nuclear IDO1 (P = 0.041), nuclear/cytoplasmic IDO1 (HR = 3.023, 95%CI: 0.585-15.61, P = 0.041) and cytoplasmic IDO1/COX2 (HR = 2.740, 95%CI: 0.764-9.831, P = 0.038) have significantly poor OS outcomes for the CRC celecoxib subgroup. In our multivariate Cox model, high coexpression of cytoplasmic IDO1/COX2 was found to be an independent predictor of poor outcome in CRC (HR = 2.218, 95%CI: 1.011-4.48, P = 0.047) and celecoxib subgroup patients (HR = 3.210, 95%CI: 1.074-9.590, P = 0.037).

CONCLUSION

Our results showed that cytoplasmic IDO1/COX2 coexpression could be used as an independent poor predictor for OS in CRC.

Key words: Prognosis; Indoleamine-2,3-dioxygenase 1; Cyclooxygenase 2; Colorectal cancer

Core tip: It was reported that indoleamine-2,3-dioxygenase 1 (IDO1) is an inhibitory factor that suppresses the T cell response to tumors. In this study, we evaluated IDO1/cyclooxygenase 2 (COX2) expression as an independent prognostic biomarker for colorectal cancer (CRC) patients. In our multivariate Cox model, high coexpression of cytoplasmic IDO1/COX2 was found to be an independent predictor of poor outcome in CRC patients and celecoxib subgroup patients. Our results showed that cytoplasmic IDO1/COX2 coexpression could be used as an independent predictor for poor overall survival in CRC.

Ma WJ, Wang X, Yan WT, Zhou ZG, Pan ZZ, Chen G, Zhang RX. Indoleamine-2,3-dioxygenase 1/cyclooxygenase 2 expression prediction for adverse prognosis in colorectal cancer. *World J Gastroenterol* 2018; 24(20): 2181-2190 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i20/2181.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i20.2181

INTRODUCTION

Colorectal cancer (CRC) is a leading cause of cancer-related death worldwide. Nearly one million cases of CRC are diagnosed worldwide each year[1,2]. Because of genetic mutations and environmental factors, CRC development is a very complex process and is determined by multistage factors[3,4]. Currently, immunotherapy has become one of the most promising treatments for CRC[5].

Recent studies have demonstrated that the tumor microenvironment plays a vital role in the progression of cancer development - *e.g.*, cancer cells, through expressing inhibitory proteins, such as PD-L1 and CTLA4, create an immunosuppressive microenvironment[6-8]. Clinical trials have shown that combining PD-1/PD-L1 with CTLA4 blockade therapy seems to be a better therapy than single blockade. However, this favorable outcome is achieved in only less than 40% of patients[9]. Other studies have confirmed that the tumor microenvironment has more inhibitory factors, including indoleamine-2,3-dioxygenase 1 (IDO1), and suppresses the T cell response to tumors. IDO1 belongs to a unique class of mammalian heme dioxygenase enzymes and is the first and rate-limiting enzyme in the degradation of the essential amino acid tryptophan, resulting in the accumulation of their metabolites such as kynurenine[9]. T cells sense low tryptophan and high kynurenine *via* mTORC and GCN2 signaling pathways to initiate an amino acid starvation response, resulting in T cell cycle arrest and cell death, and favoring the differentiation of regulatory T cells; as a result, the immune mediator is escapes in cancer[10].

In humans, IDO1 is usually expressed only in placental endothelial cells and mature dendritic cells. Activating T lymphocytes could express interferon-r in the tumor microenvironment, resulting in inducing IDO1 expression in most tissues and cell types and inhibiting T cell responses to tumor cells[11]. Many human tumors still express IDO1 through PKC and PI_3K signaling triggered by PGE2 in the absence of T cell infiltration. Constitutive expression of cyclooxygenase 2 (COX2) by MAPK signaling could induce PGE2 production[11]. Because many tumors harbor oncogenic mutations in these signaling pathways, they could express IDO1 constitutively in the absence of interferon-r. Therefore, IDO1 and COX2 are currently of great interest in cancer research as prognostic and therapeutic biomarkers of tissues and sera.

CRC has demonstrated high heterogeneity in recent years. Hence, biomarkers need to be identified and enabled to stratify the different subgroups. Similar to other tumors, such as endometrial carcinoma and liver and ovarian cancers, the IDO expression levels

are correlated with the overall survival (OS) of CRC patients[12-16]. One study showed that IDO1 expression at the invasive front was significantly associated with OS[17]. One report has hypothesized that the nuclear localization of IDO1 promotes the immunosuppression independence of enzyme activity[18]. In CRC, the level of COX2 expression was increased in up to 85 cases but not in the normal colonic epithelium. A selective COX2 inhibitor, celecoxib, could improve chemosensitivity when CRC cells are exposed to the combination with 5-FU and CPT-11[19] and could reduce hand-foot syndrome induced by capecitabine[20]. However, whether IDO1/COX2 coexpression is correlated with OS in CRC patients remains unknown.

In this study, we conducted a retrospective analysis for the potential prognostic importance of the correlation of IDO1 and COX2 in survival outcome prognosis, including their coexpression, cytoplasmic and nuclear localization of IDO1, and tumor-infiltrating lymphocytes (TILs).

MATERIALS AND METHODS

Patient characteristics

All tissues were collected from 95 patients who had undergone surgical resection from August 2008 to January 2010 at the Department of Colorectal Surgery of Sun Yat-sen University (Guangzhou, China). Patients were randomly assigned to adjuvant treatment with XELOX/capecitabine alone combined with or without celecoxib groups after surgery. All patients in the groups received celecoxib 200 mg/m^2 twice daily, given for 14 d (day 1 to day 14) of a 3-wk cycle for total of 6-8 cycles[20]. The eligibility criteria were as follows: (1) Stage Ⅱ/Ⅲ CRC eligible for adjuvant chemotherapy; (2) all tumor tissue pathological diagnoses confirmed to be CRC by a pathologist. The cases were selected consecutively based on the availability of resection tissues and follow-up data.

Immunohistochemical staining

Formalin-fixed, paraffin-embedded tumor specimens were cut into 4-μm sections. After baking at 60 ℃ for 2 h, the samples were deparaffinized in xylene and rehydrated in a series of graded ethanol. Next, the samples were incubated with 3% hydrogen peroxide for 10-15 min to block endogenous peroxidase activity. The sections were microwaved for antigen retrieval in 0.01 mol/L sodium citrate buffer (pH 6.0) for 30 min, and then were pre-incubated in 10% normal goat serum for 30 min to block nonspecific staining. The sections were then incubated with the primary rabbit anti-human IDO1 monoclonal antibody (working dilution, 1:100; Cell Signaling Technology, Danvers, MA, United States), rabbit antihuman COX2 monoclonal antibody (working dilution, 1:200; Beijing Golden Bridge Biotechnology, China), rabbit antihuman CD3 monoclonal (working dilution: 1:50; Beijing Golden Bridge Biotechnology) and mouse antihuman CD8 monoclonal (working dilution, 1:100; Beijing Golden Bridge Biotechnology) overnight at 4 ℃. Subsequently, the samples were incubated with secondary antibody (Dako, Glostrup, Denmark) at room temperature for 0.5 h.

All the stained slides were scored independently by two experienced pathologists who were blinded to the patients' identity and clinical status. H-scores of dominant staining intensity (0, 1+, 2+ and 3+) and the percentage of positive tumor cells (0 to 100%) of immunostaining were adopted for the expression data analysis. IDO1 expression was classified as high or low based on whether the H-score was above or below the score of 0.1. COX2 expression was considered high if the score was above 0.6 as the median cut-off. T cell infiltration of tumors was assessed by semiquantitative estimation of the density of CD3-positive/CD8-positive (CD3+/CD8+) cells and was scored as follows: 1+: No or sporadic CD3+/CD8+CD3þ cells; 2+: Moderate numbers of CD3+/CD8+ cells; 3+: Abundant occurrence of CD3+/CD8+ cells; and 4+: Highly abundant occurrence of CD3+/CD8+ cells[21].

Follow-up

The last date of follow-up was October 2017. All patients (51 males and 44 females) were followed up every 3 mo in the first 2 years and every 6 mo thereafter. History and physical examination should be given every 3 to 6 mo for 2 years, and then every 6 mo for a total of 5 years. A carcinoembryonic antigen (CEA) test and abdominal and pelvic ultrasound test were recommended at baseline and every 3 to 6 mo for 2 years, then every 6 mo for a total of 5 years. Colonoscopy is recommended at approximately 1 year after resection. Repeat colonoscopy is typically recommended at 3 years, and every 5 years thereafter, unless follow-up colonoscopy indicates advanced adenoma, in which case colonoscopy should be repeated in 1 year. Chest, abdominal and pelvic CT scans were recommended annually for up to 5 years. During the follow-up, 33 patients (34.7%) died of cancer-related causes. Sixty-two patients (65.3%) were still alive at the time of the last follow-up report.

Statistical analysis

The SPSS software package (version 23.0; IBM Corp, Armonk, NY, United States) and GraphPad Prism (version 7.0; GraphPad Software Inc, La Jolla, CA, United States) were used for statistical analysis. OS was defined as the time from the diagnosis of CRC to death of the patient or last date of follow-up. Chi-square test was used to assess the correlation of the IDO1 status with clinicopathologic characteristics. Survival curves were generated using the Kaplan-Meier method, and differences between curves were assessed by the log-rank test. The Cox multivariate proportional hazards regression model was used to determine the independent risk factors that influence OS. *P*-values < 0.05 were considered to be statistically significant.

RESULTS

IDO1 and COX2 expression in CRC

To elucidate the biological significance of IDO1/COX2 in

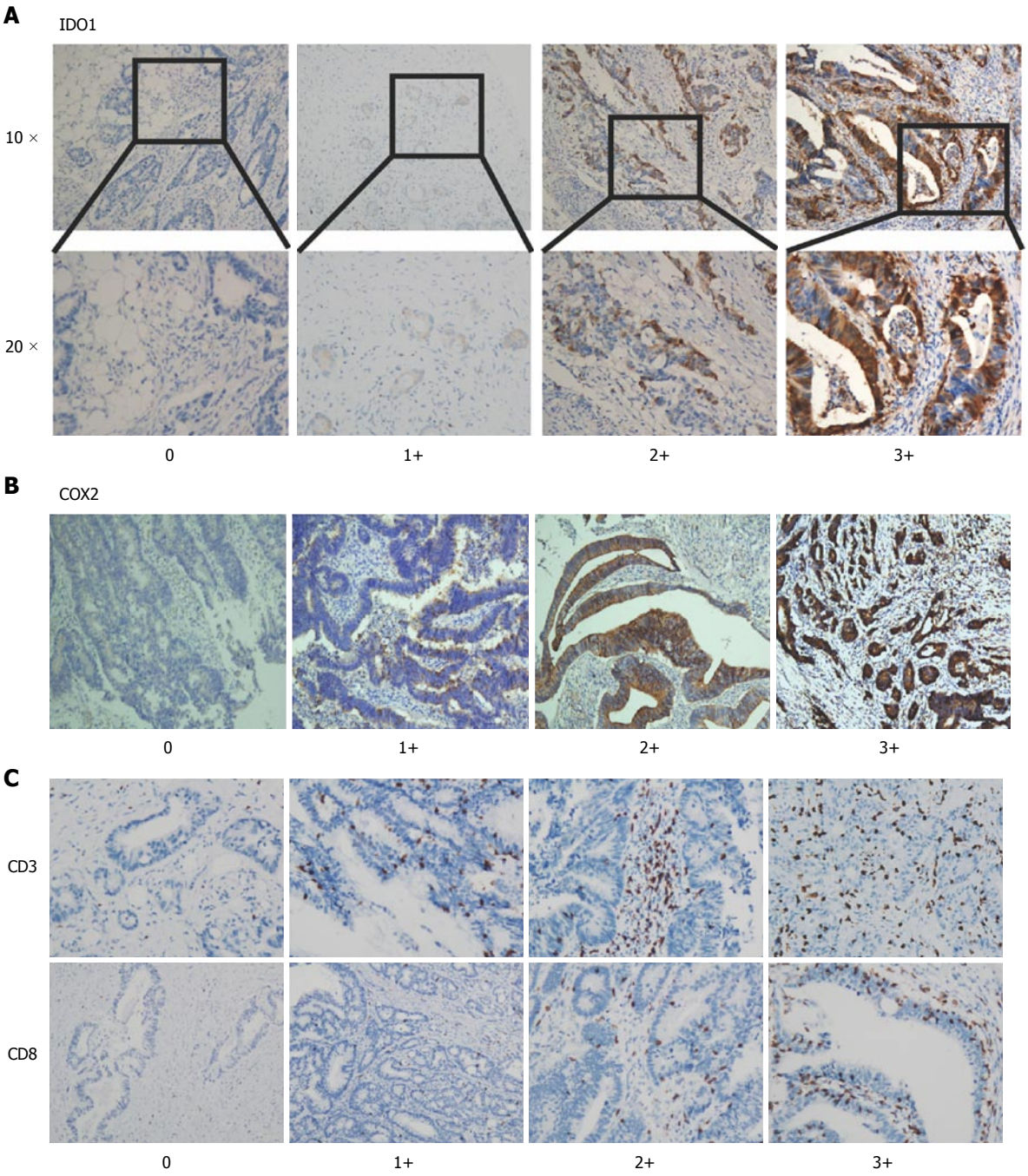

Figure 1 Indoleamine-2,3-dioxygenase 1, cyclooxygenase 2, CD3 and CD8 expression in colorectal cancer. A: Examples of the tumoral staining intensity (0, 1+, 2+ and 3+) of IDO1 in immunohistochemistry analysis; B: Examples of the tumoral staining intensity (0, 1+, 2+ and 3+) of COX2 in immunohistochemistry analysis; C: Representative examples of tumors with intraepithelial CD3 and CD8 scores (1+, 2+, 3+ and 4+). CRC: Colorectal cancer; COX2: Cyclooxygenase 2; IDO1: Indoleamine-2,3-dioxygenase 1.

CRC, especially in the CRC celecoxib subgroup, we used immunohistochemical staining to test the expression of IDO1 and COX2 in the selected 95 CRC specimens. The results showed that IDO1 expression is primarily localized in the cytoplasm within the nucleus of tumor cells (Figure 1).

Association of cytoplasmic and nuclear IDO1 expression with clinicopathological parameters in CRC patients

To gain insight into the role of the localization of IDO1 protein in CRC, we correlated cytoplasmic and nuclear IDO1 expression in the study cohort of 95 CRC patients with certain clinical and pathological factors. The expression of nuclear IDO1 was significantly correlated with body mass index (BMI) ($P < 0.001$); however, cytoplasmic IDO1 showed no relationship with BMI ($P = 0.16$). We observed no relationship between cytoplasmic and nuclear IDO1 expression and clinical factors such as sex, age, cancer (colon and rectum), tumor differentiation, T stage, N stage, CEA, cancer antigen (CA)19-9, CD3+ and CD8+ TILs, COX2, and celecoxib treatment (Tables 1 and 2).

Table 1 Correlation of cytoplasmic indoleamine-2,3-dioxygenase 1 expression with colorectal cancer clinicopathologic parameters

Characteristic	Total	Low IDO1	High IDO1	*P*-value
Sex				0.074
Male	52	22 (42.3)	30 (57.7)	
Female	43	27 (62.8)	16 (37.2)	
Age in yr				0.65
⩾ 60	30	17 (56.7)	13 (43.3)	
< 60	65	32 (49.2)	33 (50.8)	
Cancers				0.93
Colon	46	24 (52.2)	22 (47.8)	
Rectum	49	25 (51.0)	24 (49.0)	
BMI				0.16
⩾ 25	20	7 (35.0)	13 (65.0)	
< 25	75	42 (56.0)	33 (44.0)	
Tumor differentiation				0.47
Moderate and poor	78	42 (54.5)	35 (45.5)	
Well	17	7 (41.2)	10 (58.8)	
Colon cancer stage				0.52
3	20	12 (60.0)	8 (40.0)	
2	26	12 (46.2)	14 (53.8)	
T stage				0.69
4	29	14 (48.3)	15 (51.7)	
2/3	17	10 (58.8)	7 (41.2)	
N stage				0.96
1/2	20	11 (55.0)	9 (45.0)	
0	26	13 (50.0)	13 (50.0)	
Rectum cancer stage				0.67
3	24	11(45.8)	13 (54.2)	
2	25	14 (46.0)	11 (44.0)	
T stage				0.68
4	22	12 (55.6)	10 (45.4)	
2/3	27	12 (44.4)	15 (55.6)	
N stage				0.88
1/2	24	12 (50.0)	12 (50.0)	
0	25	13 (52.0)	12 (48.0)	
CEA in ng/mL				0.45
> 5	42	24 (57.1)	18 (42.9)	
⩽ 5	53	25 (47.2)	28 (42.8)	
CA19-9 in U/mL				0.22
> 37	17	6 (35.3)	11 (64.7)	
⩽ 37	78	43 (55.1)	35 (44.9)	
CD3 TILs				0.27
High	36	22 (61.1)	14 (38.9)	
Low	59	28 (47.5)	31 (42.5)	
CD8 TILs				0.96
High	22	12 (54.5)	10 (45.5)	
Low	73	38 (52.5)	35 (47.5)	
COX2				0.92
High	48	26 (54.2)	22 (45.8)	
Low	47	24 (51.1)	23 (48.9)	
Treatment group				0.58
Celecoxib	44	25 (56.8)	19 (43.2)	
Non-celecoxib	51	25 (49.0)	26 (51.0)	

Data are presented as *n* or *n* (%). BMI: Body mass index; CA: Cancer antigen; CEA: Carcinoembryonic antigen; COX2: Cyclooxygenase 2; CRC: Colorectal cancer; IDO1: Indoleamine-2,3-dioxygenase 1; TILs: Tumor-infiltrating lymphocytes.

Table 2 Correlation of nuclear indoleamine-2,3-dioxygenase 1 expression with colorectal cancer clinicopathologic parameters

Characteristic	Total	Low IDO1	High IDO1	*P*-value
Sex				0.70
Male	51	39 (76.5)	12 (33.5)	
Female	44	36 (81.8)	8 (19.2)	
Age in yr				0.92
> 60	30	24 (80.0)	6 (20.0)	
⩽ 60	65	51 (78.5)	14 (21.5)	
Cancers				0.52
Colon	46	38 (82.6)	18 (17.4)	
Rectum	49	37 (75.5)	12 (24.5)	
BMI				< 0.001
> 25	20	9 (45.0)	11 (55.0)	
⩽ 25	75	66 (88.0)	9 (12.0)	
Tumor differentiation				0.87
Moderate and poor	78	60 (76.9)	18 (23.1)	
Well	17	14 (82.3)	3 (17.7)	
Conlon cancer stage				0.98
3	20	17 (85.0)	3 (15.0)	
2	26	21 (80.8)	5 (19.2)	
T stage				0.71
4	29	23 (19.3)	6 (20.7)	
2/3	17	15 (88.2.)	2 (11.8)	
N stage				0.98
1/2	20	16 (80.0)	4 (20.0)	
0	26	22 (84.6)	4 (15.4)	
Rectum cancer stage				0.44
3	24	17 (70.8)	7 (29.2)	
2	25	21 (84.0)	4 (16.0)	
T stage				0.94
4	22	16 (72.7)	6 (27.3)	
2/3	27	21 (77.8)	6 (22.2)	
N stage				0.28
1/2	24	16 (66.7)	8 (33.3)	
0	25	21(84.0)	4 (16.0)	
CEA in ng/mL				0.37
> 5	42	35 (83.3)	7 (16.7)	
⩽ 5	53	39 (73.6)	14 (26.4)	
CA19-9 in U/mL				0.78
> 37	81	41 (50.6)	40 (49.4)	
⩽ 37	17	8 (47.1)	9 (52.9)	
CD3 TILs				0.96
High	36	28 (77.8)	8 (22.2)	
Low	59	47 (79.7)	12 (20.3)	
CD8 TILs				0.26
High	22	15 (68.2)	7 (31.8)	
Low	73	60 (82.2)	13 (17.8)	
COX2				0.84
High	48	38 (79.2)	10 (20.8)	
Low	47	37 (78.7)	10 (21.3)	
Treatment group				0.16
Celecoxib	44	38 (86.4)	6 (13.6)	
Non-celecoxib	51	37 (72.5)	14 (27.5)	

Data are presented as *n* or *n* (%). BMI: Body mass index; CA: Cancer antigen; CEA: Carcinoembryonic antigen; COX2: Cyclooxygenase 2; CRC: Colorectal cancer; IDO1: Indoleamine-2,3-dioxygenase 1; TILs: Tumor-infiltrating lymphocytes.

Correlation of IDO1/COX2 protein expression with poor prognosis in CRC

We analyzed the correlation between IDO1 and traditional clinicopathologic parameters with patients' outcomes by univariate analysis. We also performed analyses to determine whether IDO1 and COX2 expression and localization represent potential independent predictors for the OS outcome in CRC patients. We observed that cytoplasmic IDO1 and COX2 expression could not predict OS outcomes in our univariate analysis (cytoplasmic IDO1: $P = 0.10$; COX2: $P = 0.51$). However, nuclear IDO1 ($P = 0.039$), nuclear/cytoplasmic IDO1 (hazard ratio (HR) = 2.044, 95% confidence interval (CI): 0.871-4.798, $P = 0.039$),

Table 3 Univariate analysis of the correlation of clinicopathological parameters with overall survival in patients with colorectal carcinoma

	HR	95%CI	*P* value
Sex, male *vs* female	0.750	0.399-1.411	0.37
Age in yr, ≤ 60 *vs* > 60	0.899	0.472-1.714	0.74
Cancer, colon *vs* rectum	1.279	0.712-2.296	0.41
BMI, > 25 *vs* ≤ 25	1.579	0.697-3.579	0.21
Tumor differentiation, moderate and poor *vs* well	2.798	1.373-5.702	0.039
Stage, 3 *vs* 2	1.003	0.534-1.882	0.99
T stage, T4 *vs* T2/3	1.418	0.755-2.664	0.27
N stage, N1/2 *vs* N0	1.005	0.536-1.887	0.99
CEA in ng/mL, > 5 *vs* ≤ 5	2.137	1.141-4.004	0.025
CA19-9 in U/mL, > 37 *vs* ≤ 37	1.262	0.547-2.911	0.56
CD3 TILs, high *vs* low	1.195	0.649-2.198	0.55
CD8 TILs, high *vs* low	2.096	0.975-4.504	0.018
Nuclear IDO1, high *vs* low	2.044	0.871-4.798	0.039
Cytoplasmic IDO1, high *vs* low	1.690	0.901-3.173	0.10
Nuclear and cytoplasmic IDO1, high *vs* low	2.044	0.871-4.798	0.039
COX2, high *vs* low	1.235	0.659-2.314	0.51
Nuclear IDO1/COX2, Ⅳ *vs* Ⅰ / Ⅱ / Ⅲ	3.048	0.868-10.7	0.0049
Cytoplasmic IDO1/COX2, Ⅳ *vs* Ⅰ / Ⅱ / Ⅲ	2.109	0.976-4.558	0.022
Treatment group, celecoxib *vs* non-celecoxib	0.943	0.489-1.826	0.86

BMI: Body mass index; CA: Cancer antigen; CEA: Carcinoembryonic antigen; CI: Confidence interval; COX2: Cyclooxygenase 2; CRC: Colorectal carcinoma; HR: Hazard ratio; IDO1: Indoleamine-2,3-dioxygenase 1; TILs: Tumor infiltrating lymphocytes.

nuclear IDO1/COX2 (HR = 3.048, 95%CI: 0.868-10.7, *P* = 0.0049), cytoplasmic IDO1/COX2 (HR = 2.109, 95%CI: 0.976-4.558, *P* = 0.022), tumor differentiation (HR = 2.798, 95%CI: 1.373-5.702, *P* = 0.039), CEA (HR = 2.137, 95%CI: 1.141-4.004, *P* = 0.025), and CD8 TILs (HR = 2.096, 95%CI: 0.975-4.504, *P* = 0.018) (Table 3) yielded significantly poor OS outcomes in CRC patients (Figure 2B-G, Supplementary Figure 1E) but not with other clinicopathologic parameters such as sex, age, BMI, T stage, N stage, CA19-9 and CD3+ TILs, including whether celecoxib was used or not (Figure 2A, 2C, 2H, Supplementary Figure 1A-D, 1F-J).

We also performed multivariate Cox modeling to analyze whether IDO1/COX2 represent potential independent predictors for the OS outcome in CRC patients. Combined cytoplasmic IDO1/COX2 coexpression analysis yielded a stronger predictor index, with HR = 2.218 (95%CI: 1.011-4.48, *P* = 0.047) in the $IDO1^{High}/COX2^{High}$ group, and tumor differentiation was significantly correlated with OS (HR = 3.473, 95%CI: 1.201-10.046, *P* = 0.022) (Table 4) but not nuclear IDO1, cytoplasmic IDO1, nor combined nuclear IDO1/COX2 expression. Our results revealed that cytoplasmic IDO1/COX2 coexpression and tumor differentiation were independent predictors for poor OS in CRC.

Correlation of IDO1/COX2 protein expression with poor prognosis in the CRC celecoxib subgroup

We also performed analyses to determine whether IDO1 and COX2 expression and localization represent potential independent predictors for OS outcome in CRC patients. We observed that cytoplasmic IDO1 and COX2 expression could not predict OS outcomes in univariate analysis (cytoplasmic IDO1: *P* = 0.31; COX2: *P* = 0.25). However, nuclear IDO1 (*P* = 0.041), nuclear/cytoplasmic IDO1 (HR = 3.023, 95%CI: 0.585-15.61, *P* = 0.041), cytoplasmic IDO1/COX2 (HR = 2.740, 95%CI: 0.764-9.831, *P* = 0.038) (Table 5), tumor differentiation (HR = 7.396, 95%CI: 2.749-19.90, *P* = 0.021) and CD8 TILs (HR = 2.821, 95%CI: 0.774-10.29, *P* = 0.026) have significantly poor OS outcomes for the CRC celecoxib subgroup (Figure 3B, 3D, 3F, 3H and 3I) but not with other clinicopathologic parameters such as sex, age, BMI, T stage, N stage, CEA, CA19-9 and CD3+ TILs (Figure 3A, 3C, 3E and 3G, Supplementary Figure 2A-I).

We further performed the multivariate Cox modeling to analyze whether IDO1/COX2 represents potential independent predictors for OS outcome in the CRC celecoxib subgroup. Combined cytoplasmic IDO1/COX2 coexpression analysis yielded a stronger predictor index, with HR = 3.210 (95%CI: 1.074-9.590, *P* = 0.037) in the $IDO1^{High}/COX2^{High}$ group, and tumor differentiation was significantly correlated with OS (HR = 11.962, 95%CI: 1.526-23.787, *P* = 0.018) (Table 6) but not nuclear IDO1, cytoplasmic IDO1, nor combined nuclear IDO1/COX2 expression. Our results revealed that cytoplasmic IDO1/COX2 coexpression and tumor differentiation were independent poor predictors of OS in the CRC celecoxib subgroup.

DISCUSSION

Current immunotherapy has been achieving very effective and promising results, especially for stage IV disease. However, more than 50% of these patients who need more new therapies will progress with resistance to immunotherapy[22]. IDO1 is associated with T cell apoptosis through depleting tryptophan in the tumor microenvironment. Therefore, IDO1 inhibitors have emerged as new options for cancer therapy. However, a

Table 4 Multivariate analysis of the correlation of indoleamine-2,3-dioxygenase 1 with overall survival in patients with Colorectal cancer

	HR	95%CI	*P*-value
Cytoplasmic IDO1 and COX2, Ⅳ *vs* Ⅰ / Ⅱ / Ⅲ	2.218	1.011-4.48	0.047
Tumor differentiation, poor and moderate *vs* well	3.473	1.201-10.046	0.022

CI: Confidence interval; COX2: Cyclooxygenase 2; HR: Hazard ratio; IDO1: Indoleamine-2,3-dioxygenase 1.

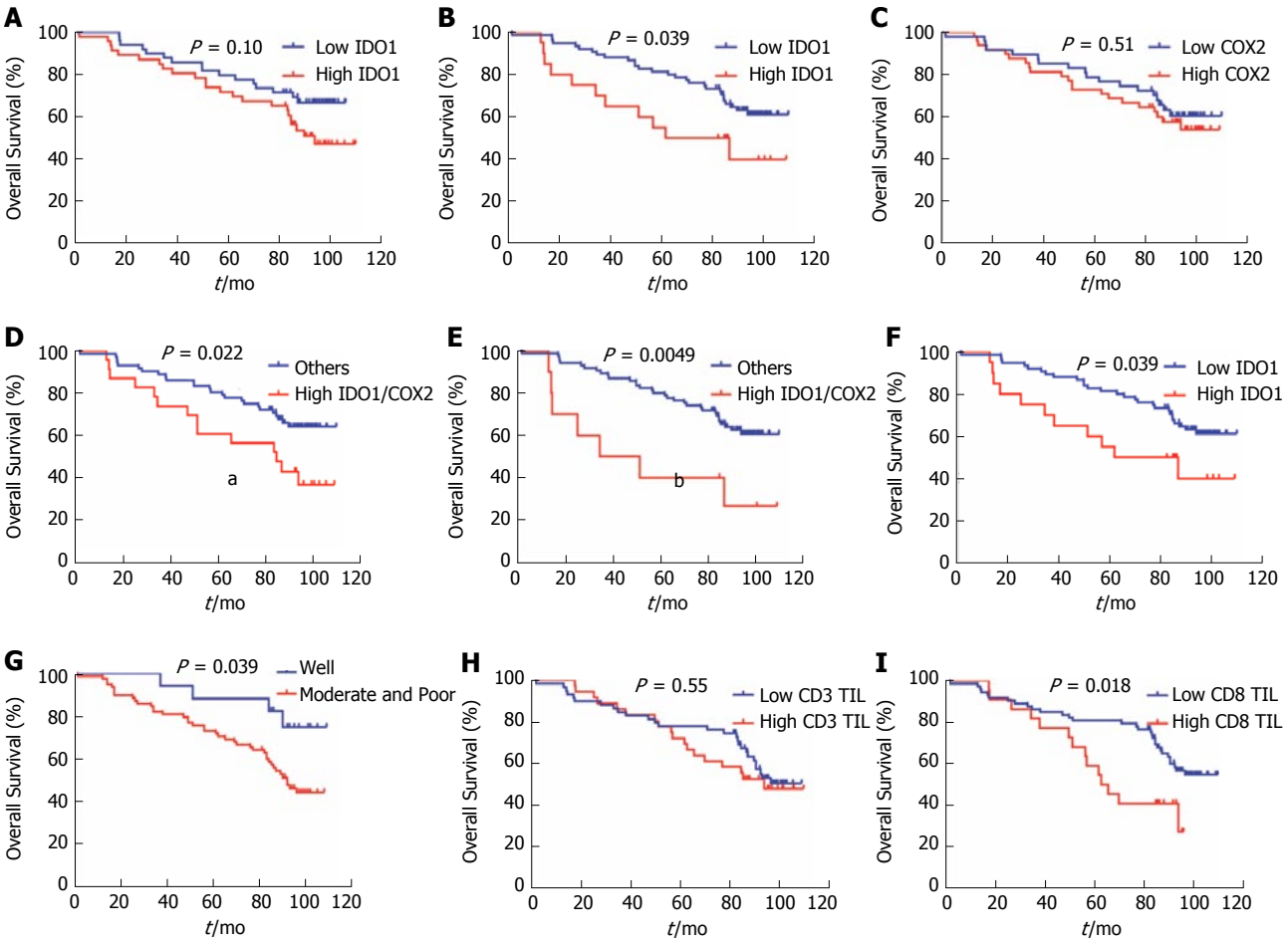

Figure 2 Correlation of Indoleamine-2,3-dioxygenase 1/cyclooxygenase 2 protein expression with a poor prognosis in colorectal cancer. A-C: Correlation between nuclear or cytoplasmic IDO1 and COX2 expression with CRC patient OS. Survival curves were generated using the Kaplan-Meier method, and differences between survival curves were estimated by the log-rank test. Nuclear IDO1 showed a statistically significant correlation with OS; D-E: Correlation between the different expression levels of nuclear and cytoplasmic IDO1/COX2 and OS in CRC patients. Group Ⅰ: IDO1^{Low}COX2^{Low}; Group Ⅱ: IDO1^{High}COX2^{Low}; Group Ⅲ: IDO1^{Low}COX2^{High}; Group Ⅳ: IDO1^{High}COX2^{High}. The association of the four groups (Ⅳ *vs* Ⅰ / Ⅱ / Ⅲ) with OS was significant ($P < 0.05$); F: Combined analysis of nuclear and cytoplasmic IDO1 and its correlation with OS in CRC. The association of nuclear and cytoplasmic IDO1 expression with OS was significant ($P < 0.05$); G: Correlation between tumor differentiation and OS in CRC. The association of tumor differentiation (moderate and poor vs well) with OS was significant ($P < 0.05$); H-I: Correlation between CD3 TILs and CD8 TILs and OS in CRC; H: CD3 TILs ($P > 0.05$); I: CD8 TILs ($P < 0.05$). CRC: Colorectal cancer; COX2: Cyclooxygenase 2; IDO1: Indoleamine-2,3-dioxygenase 1; OS: Overall survival; TILs: Tumor-infiltrating lymphocytes.

recent study suggested the alternative hypothesis that nuclear IDO1 promotes immunosuppression instead of enzyme activity[18]. In previous studies, high IDO expression in CRC has been found to be associated with the presence of metastatic disease and outcome and a reduction in CD3-positive TILs, revealing the important role in therapeutic blockade for this disease[12,17]. In up to 85% of CRC patients, COX2 is highly expressed but not in normal colonic epithelium. Celecoxib is a COX2 inhibitor used in the treatment regimen for CRC; previous studies have demonstrated celecoxib in combination with chemotherapy to overcome resistance in therapy-refractory cancer cells *in vitro* and *in vivo*[19]. However, clinical studies have not been clarified to show the role of celecoxib in CRC patients and its potential prognostic importance.

In the present study, we evaluated CRC patients treated with or without celecoxib. We found no significant relationship with IDO1 or COX2 expression and OS in patients treated with or without celecoxib. However, our discovery revealed that cytoplasmic IDO1 and COX2 were correlated with OS in patients treated with or

Table 5 Univariate analysis of the correlation of clinicopathological parameters with overall survival in CRC celecoxib subgroup

	HR	95%CI	*P* value
Sex, male *vs* female	0.854	0.329-2.219	0.74
Age in yr, ≤ 60 *vs* > 60	1.249	0.432-3.609	0.70
Cancer, colon *vs* rectum	1.034	0.420-2.543	0.94
BMI, > 25 vs ≤ 25	1.328	0.351-5.020	0.71
Tumor differentiation, moderate and poor *vs* well	7.396	2.749-19.90	0.021
Stage, 3 *vs* 2	1.075	0.415-2.782	0.88
T stage, T4 *vs* T2/3	1.389	0.537-3.596	0.50
N stage, N1/2 *vs* N0	1.075	0.415-2.782	0.88
CEA in ng/mL, > 5 *vs* ≤ 5	1.934	0.743-5.033	0.21
CA19-9 in m/L, > 37 *vs* ≤ 37	1.551	0.431-5.575	0.43
CD3 TILs, high *vs* low	1.02	0.414-2.510	0.97
CD8 TILs, high *vs* low	2.821	0.774-10.29	0.026
Nuclear IDO1, high *vs* low	3.023	0.585-15.61	0.041
Cytoplasmic IDO1, high *vs* low	1.623	0.617-4.267	0.31
Nuclear and cytoplasmic IDO1, high *vs* low	3.023	0.585-15.61	0.041
COX2, high *vs* low	1.746	0.672-4.541	0.25
Nuclear IDO1/COX2, Ⅳ *vs* Ⅰ / Ⅱ / Ⅲ	1.885	0.279-12.76	0.38
Cytoplasmic IDO1/COX2, Ⅳ *vs* Ⅰ / Ⅱ / Ⅲ	2.740	0.764-9.831	0.038

BMI: Body mass index; CA: Cancer antigen; CEA: Carcinoembryonic antigen; CI: Confidence interval; COX2: Cyclooxygenase 2; CRC: Colorectal carcinoma; HR: Hazard ratio; IDO1: Indoleamine-2,3-dioxygenase 1; TILs: Tumor infiltrating lymphocytes.

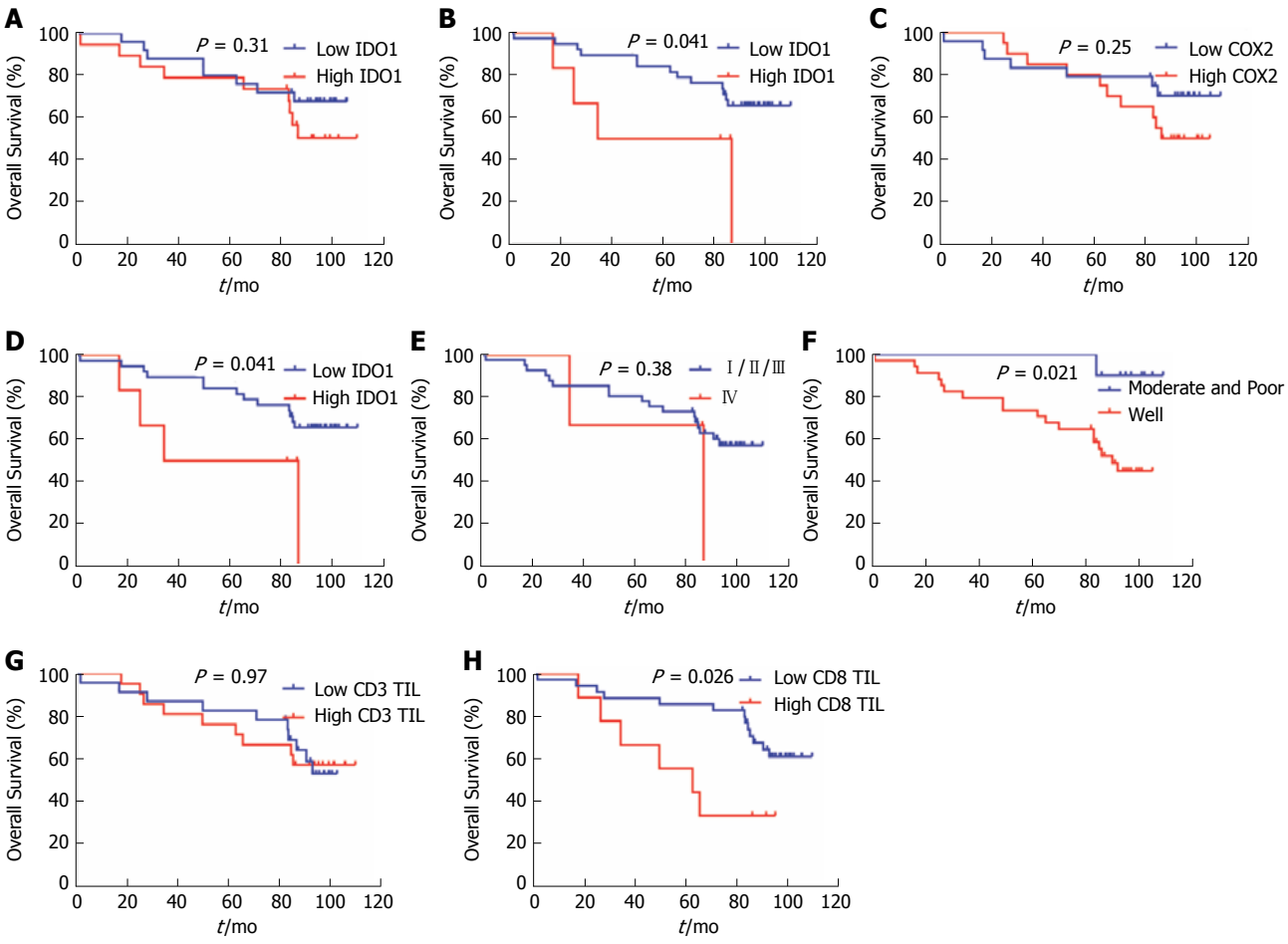

Figure 3 Correlation of indoleamine-2,3-dioxygenase 1/cyclooxygenase 2 protein expression with a poor prognosis in the colorectal cancer celecoxib subgroup. A-D: Correlation between nuclear or cytoplasmic IDO1 and COX2 expression with OS in the CRC celecoxib subgroup. Survival curves were generated using the Kaplan-Meier method, and differences between survival curves were estimated by the log-rank test. Nuclear IDO1 and nuclear and cytoplasmic IDO1 showed a statistically significant correlation with OS; E: Correlation between different expression levels of nuclear and cytoplasmic IDO1/COX2 with the OS of the CRC celecoxib subgroup. Group Ⅰ: $IDO1^{Low}COX2^{Low}$; Group Ⅱ: $IDO1^{High}COX2^{Low}$; Group Ⅲ: $IDO1^{Low}COX2^{High}$; Group Ⅳ: $IDO1^{High}COX2^{High}$. The association of four groups (Ⅳ *vs* Ⅰ / Ⅱ / Ⅲ) with OS is not significant ($P > 0.05$); F: Correlation between tumor differentiation and OS in CRC. The association of tumor differentiation (moderate and poor *vs* well) with OS is significant ($P < 0.05$); G-H: Correlation between CD3 TILs and CD8 TILs with CRC OS; G: CD3 TILs ($P > 0.05$); H: CD8 TILs ($P < 0.05$). CRC: Colorectal cancer; COX2: Cyclooxygenase 2; IDO1: Indoleamine-2,3-dioxygenase 1; OS: Overall survival; TILs: Tumor-infiltrating lymphocytes.

Table 6 Multivariate analysis of the correlation of indoleamine-2,3-dioxygenase 1 with overall survival in Colorectal cancer celecoxib subgroup

	HR	95%CI	*P*-value
Cytoplasmic IDO1 and COX2, Ⅳ *vs* Ⅰ / Ⅱ / Ⅲ	3.210	1.074-9.590	0.037
Tumor differentiation, poor and moderate *vs* well	11.962	1.526-23.787	0.018

CI: Confidence interval; COX2: Cyclooxygenase 2; HR: Hazard ratio; IDO1: Indoleamine-2,3-dioxygenase 1.

without celecoxib. Additionally, our data further found that nuclear IDO1 and COX2 were not correlated with OS in patients of either group. However, one recent study showed that nuclear IDO1 plays a more important role in CRC instead of enzyme activity. From our data, nuclear IDO1 could not be an independent prognostic factor for CRC patients. Some other unknown factors in the nucleus might combine to nuclear IDO1, possibly influencing the OS of CRC patients. These patients in our study have not been treated with IDO1 inhibitors. Therefore, whether nuclear expression affects IDO1 inhibitors is unclear.

Constitutive IDO1 expression is dependent on an autocrine loop of PGE2 production through activating the PI3K and PKC pathways and subsequent activation of IDO1 transcription by factors such as ETV4. PGE2 production mediates the expression of COX2. However, in our study, we found that IDO1 or COX2 expression was not correlated with OS. Three explanations are possible. First, CRC patients were treated with celecoxib only for no more than 6 mo. COX2 might still influence the expression of IDO1, which would negatively regulate effector T cells. Second, another signaling pathway might activate IDO1 expression in CRC patients. Third, these patients were treated with celecoxib but not combined with IDO1 inhibitors.

There are some limitations in our current study. This study was a retrospective study, with its intrinsic associated limitations. Second, although our cohort size consists of well-annotated celecoxib groups, its number is still modest. Third, to minimize bias and immunohistochemistry methodological limitations, we have herein adopted rigorous standardized assay methods in our study. All immunohistochemistry scores were affirmed by two blinded, well-trained clinical pathologists working independently. Furthermore, a larger clinical sample cohort size would be valuable to validate our results, and more chemotherapy-resistant patients need to be considered.

The results of the current study demonstrate that the coexpression of cytoplasmic IDO1 and COX2 plays a key role in survival prognosis for CRC patients; IDO1 or COX2, nuclear IDO1 and COX2 alone may not serve as a feasible biomarker for prognostic prediction. Therefore, localization of IDO1 and COX2 may serve as a better biomarker to predict CRC patient OS.

ARTICLE HIGHLIGHTS

Research background

Colorectal cancer (CRC) is a leading cause of cancer-related death worldwide. Because of genetic mutations and environmental factors, CRC development is a very complex process and is determined by multistage factors. Currently, immunotherapy has become one of the most promising treatments for CRC. However, whether indoleamine-2,3-dioxygenase 1/cyclooxygenase 2 (IDO1/COX2) coexpression is correlated with overall survival (OS) in CRC patients remains unknown.

Research motivation

CRC has demonstrated high heterogeneity in recent years. Recent studies have demonstrated that IDO1 can suppress the T cell response to tumors. A selective COX2 inhibitor, celecoxib, could improve chemosensitivity when CRC cells are exposed to the combination of 5-FU and CPT-11 and could reduce hand-foot syndrome induced by capecitabine. In this study, we conducted a retrospective analysis for the potential prognostic importance of the correlation of IDO1 and COX2 in survival outcome prognosis, including their coexpression, cytoplasmic and nuclear localization of IDO1, and tumor-infiltrating lymphocytes.

Research objectives

This study aimed to clarify the potential significance of IDO1/COX2 as a prognostic biomarker in CRC *in vitro*.

Research methods

Immunohistochemical staining of IDO1 and COX2 was performed in a clinical cohort consisting of 96 CRC cases. Expression of IDO1 and COX2 was correlated with clinicopathological indicators and the clinical outcome of CRC patients.

Research results

In the CRC group, combined cytoplasmic IDO1/COX2 coexpression analysis yielded a stronger predictor index, with hazard ratio (HR) = 2.218 (95% confidence interval (CI): 1.011-4.48, $P = 0.047$) in the $IDO1^{High}/COX2^{High}$ group, and tumor differentiation was significantly correlated with OS (HR = 3.473, 95%CI: 1.201-10.046, $P = 0.022$) but not nuclear IDO1, cytoplasmic IDO1, nor combined nuclear IDO1/COX2 expression. Our results revealed that cytoplasmic IDO1/COX2 coexpression and tumor differentiation were independent predictors for poor OS in CRC.

In the CRC celecoxib subgroup, combined cytoplasmic IDO1/COX2 coexpression analysis yielded a stronger predictor index, with HR = 3.210 (95%CI: 1.074-9.590, $P = 0.037$) in the $IDO1^{High}/COX2^{High}$ group, and tumor differentiation was significantly correlated with OS (HR = 11.962, 95%CI: 1.526-23.787, $P = 0.018$) but not nuclear IDO1, cytoplasmic IDO1, nor combined nuclear IDO1/COX2 expression.

Research conclusions

The results of the current study demonstrate that the coexpression of cytoplasmic IDO1 and COX2 plays a key role in survival prognosis in CRC patients.

Research perspectives

IDO1 could be a novel therapeutic target for human CRC, especially as a biotarget of immunotherapy.

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ORIGINAL ARTICLE

Clinical Trials Study

Regulatory polymorphism of CXCL10 rs1439490 in seronegative occult hepatitis C virus infection

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Abstract

AIM

To examine the relationship between the single nucleotide polymorphism CXCL10 rs1439490 and seronegative occult hepatitis C virus (HCV) infection (OCI).

METHODS

One hundred and three cases of seronegative OCI and 155 cases of seropositive chronic HCV infection (CHC) were diagnosed at five Liver Centers in Northeastern China, from 2012 to 2016. CXCL10 rs1439490, rs1440802, and IL-28B rs12979860 were analyzed by sequencing. Serum CXCL10 was measured by ELISA. Intrahepatic CXCL10 was determined by quantitative PCR and immunohistochemical semi-quantitative scoring. Liver necroinflammation and fibrosis were scored according to the METAVIR system.

RESULTS

CXCL10 rs1439490 G/G was more prevalent in OCI patients (n = 93/103; 90.3%) than in CHC patients (n = 116/155; 74.8%; P = 0.008). OCI patients had lower serum CXCL10 levels than CHC patients (192.91 ± 46.50 pg/mL *vs* 354.78 ± 102.91 pg/mL, P < 0.0001). Of IL-28B rs12979860 C/C patients, OCI patients with rs1439490 G/G had lower serum and liver levels of CXCL10 and lower levels of liver necroinflammation and fibrosis-than non-G/G patients. OCI patients had higher alanine aminotransferase normalization rates after Peg-interferon treatment than CHC patients (P < 0.05) and serum CXCL10 decreased significantly (P < 0.0001). Liver necroinflammation and fibrosis were alleviated in 8 OCI patients after treatment. Multivariate analysis indicated that rs1439490 G/G significantly influenced the occurrence of OCI in HCV infection (OR = 0.31, 95%CI: 0.15-0.66, P = 0.002).

CONCLUSION

CXCL10 rs1439490 G/G is positively associated with OCI in HCV infection and antiviral outcome.

Key words: Occult hepatitis C virus infection; CXCL10; Single nucleotide polymorphisms; rs1439490

Core tip: We demonstrated that CXCL10 rs1439490 G/G was more prevalent in patients with seronegative occult hepatitis C virus infection (OCI) than in those with seropositive chronic hepatitis C virus (HCV) infection (CHC). Rs1439490 G/G OCI patients had lower serum and liver levels of CXCL10, and lower levels of liver necroinflammation and fibrosis than non-G/G patients. OCI patients had higher alanine aminotransferase normalization rates after Peg-interferon treatment than CHC patients and serum CXCL10 decreased significantly. We, for the first time, showed that CXCL10 rs1439490 G/G may be positively associated with OCI in HCV infection and antiviral outcome.

Wang X, Wang S, Liu ZH, Qi WQ, Zhang Q, Zhang YG, Sun DR, Xu Y, Wang HG, Li ZX, Cong XL, Zhao P, Zhou CY, Wang JB. Regulatory polymorphism of CXCL10 rs1439490 in seronegative occult hepatitis C virus infection. *World J Gastroenterol* 2018; 24(20): 2191-2202 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i20/2191.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i20.2191

INTRODUCTION

Occult hepatitis C virus (HCV) infection (OCI) involves the detection of intrahepatic HCV RNA by percutaneous transhepatic liver biopsy in patients with long-standing liver dysfunction who are seronegative for anti-HCV antibodies and RNA[1]. OCI was proposed as a subtype of chronic HCV infection (CHC)[2]. It was found to occur in 8.9% of patients with cryptogenic hepatic disease[3], 1.27% of infectious liver disease-free subjects[4], and in patients without conventional HCV markers but abnormal liver enzyme levels, or healthy subjects with normal alanine aminotransferase (ALT) levels and no clinical evidence of liver disease[1,3-12].

To date, the pathogenesis of OCI remains unclear. It is unknown why OCI patients do not produce anti-HCV antibodies after exposure to HCV and why serum HCV RNA is not detectable. Low detection sensitivity has been implicated as a major reason for OCI[13], which may explain the existence of occult infection in anti-HCV seropositive and HCV RNA seronegative individuals. Mutant HCV strains that may subvert the conventional HCV assays have also been implicated in OCI[14]. However, the 3rd generation HCV antibody detection assays cover most HCV structural and nonstructural antigens and achieve up to 99% sensitivity[15]. Instead, OCI may be the result of "sporadic" exposure to trace amounts of HCV[16] that generate insufficient T cell activation and B cell responses against infection. Consequently, serum anti-

HCV activity cannot be detected by current clinical methods. Indeed, persistent low levels of virus-specific T-cell responses have been identified in OCI patients[17,18]. However, this host immune response can only partially suppress HCV replication, but cannot eliminate the virus or viral antigens. Thus, HCV RNA remains detectable in the liver – the primary target tissue.

Patients with OCI have distinct clinical outcomes from those with CHC even of the same genotypes, indicating the role of host factors in OCI pathogenesis. The interleukin-28B (IL-28B) locus has been associated with HCV outcomes and IL-28B C/C was shown to occur more often in OCI than in CHC patients[19]. In OCI patients, intrahepatic HCV RNA load was significantly lower in those with the IL-28B C/C genotype than in those with C/T or T/T genotypes[19]. Thus, IL-28B polymorphisms may affect endogenous IFN-λ levels and be associated with low viral replication in some patients. However, interferon (IFN) has also been shown not to play a determining role in OCI occurrence, and IL-28B C/C OCI patients were found to have lower serum levels of CXC chemokine ligand 10 (CXCL10) than IL-28B C/C CHC patients[19]. Therefore, regulation of OCI and the associated disease progression likely involves additional host immune factors.

The importance of CXCL10 expression during chronic hepatitis B virus (HBV) infection has recently been emphasized. Two single nucleotide polymorphisms (SNPs) of CXCL10 (G-201A and C-1513T) were reported to have high allele frequency in chronic HBV infected Chinese populations. The polymorphism G-201A in the CXCL10 promoter was also implicated in the genetic variation underlying the susceptibility to chronic HBV infection progression[20]. G-201A is located within the CXCL10 promoter region and is proximal to the NF-κB1/2 binding sites. The G-201A SNP is associated with the expression of CXCL10 in peripheral blood mononuclear cells (PBMC), underpinning the mechanism of chronic HBV disease progression. Based on this large cohort study, and the observation that both HCV and HBV promote the development of hepatic lesions and fibrosis by inducing inflammatory infiltration rather than by damaging hepatocytes directly, we hypothesized that CXCL10 G-201A may also affect the disease manifestation of CHC. However, to date, there is no such report.

In this study, we examined the expression frequency of CXCL10 G-201A (rs1439490), C-1513T (rs1440802) and IL-28B rs12979860 SNPs in OCI and CHC patients to investigate whether these polymorphisms are associated with OCI. In addition, we further analyzed the correlation of these SNPs with the serum and liver levels of CXCL10 and liver HCV RNA levels in OCI patients.

MATERIALS AND METHODS

Patients

A total of 1796 patients with liver dysfunction and/or radiographic abnormalities of unknown etiology underwent liver biopsy between 2012 and 2016 at five hospitals in Northeastern China (China-Japan Union Hospital of Jilin University, People's Hospital of Jilin City, Fourth Affiliated University of Harbin Medical University, People's Hospital of Hunchun City, and the Second People's Hospital of Daqing City). All patients were Han Chinese. Subjects seronegative for anti-HCV antibodies and HCV RNA, but with detectable intrahepatic HCV RNA were included in the OCI group (n = 103). One hundred and fifty-five normal CHC patients who underwent liver biopsy prior to antiviral therapy during the same period were included in the CHC control group. Informed consent forms were obtained from all patients. The study was approved by the Institutional Review Boards of the individual centers (registration number: ChiCTR-ONRC-12002207).

Inclusion/exclusion criteria

OCI inclusion criteria were as follows: (1) Serum anti-HCV antibodies and HCV RNA negative in 3 consecutive tests within at least 3 mo, and persistent liver dysfunction and/or radiographic abnormalities; (2) HCV RNA/HBV DNA seronegative after ultracentrifugation and undetectable in PBMC; and (3) HCV RNA-positive in liver tissue. CHC inclusion criteria were as follows: (1) Serum anti-HCV antibodies and HCV RNA positive, and diagnosed with CHC in accordance with the EASL guidelines[21]; and (2) consent to receive hepatic histological evaluation prior to anti-HCV treatment.

Exclusion criteria were as follows: (1) Occult HBV infection, drug-induced liver disease, fatty liver disease, autoimmune liver disease, inherited metabolic liver disease after liver biopsy; (2) co-infection with other types of hepatitis (A, D, E), Epstein-Barr virus, cytomegalovirus, or human immunodeficiency virus; (3) chronic HCV infection complicated with decompensated cirrhosis or primary liver cancer; (4) severe heart, brain or kidney complications; and (5) received or receiving pegylated IFN (Peg-IFN) plus ribavirin (RBV) or IFN treatment.

SNP analyses of CXCL10 rs1439490, rs1440802, and IL-28B rs12979860

Peripheral blood samples were collected from the patients, placed in anticoagulant EDTA-treated tubes, and genomic DNA was extracted using a Puregene SK8224 DNA isolation kit from Sangon Biotech (Shanghai, China), according to the manufacturer's instructions. The primers targeting specific fragments were designed and synthesized by Sangon Biotech: CXCL10 promoter region G-201A rs1439490[20]: Forward: 5'-TTCAGTAACATAAACCCCAACAA-3'; Reverse: 5'-CACAAAGGAAGACAATAAGGGAG-3'. CXCL10 promoter region C-1513T rs1440802: Forward[20]: 5'-CTC ACTTTGTCTCACCAATCTCA-3'; Reverse: 5'-CAGAGAA ATGAGAGACCTAAGTGTG-3'. IL-28B rs12979860[22]: Forward: 5'-CCTCTGCACAGTCTGGGATTC-3'; Reverse: 5'-GCTCAGGGTCAATCACAGAAG-3'.

Table 1 Algorithm for evaluation of histological activity

Piecemeal necrosis	+	Lobular necrosis	=	Histological activity score
0 (none)		0 (none or mild)		0 (none)
0		1 (moderate)		1 (mild)
0		2 (severe)		2 (moderate)
1 (mild)		0, 1		1
1		2		2
2 (moderate)		0, 1		2
2		2		3 (severe)
3 (severe)		0, 1, 2		3

Table 2 Fibrosis scoring

Description	Score
No fibrosis	0
Stellate enlargement of portal tract but without septa formation	1
Enlargement of portal tract with rare septa formation	2
Numerous septa without cirrhosis	3
Cirrhosis	4

Serum CXCL10 levels

Serum CXCL10 levels were measured by human CXCL10 Quantikine ELISA (R&D, Minneapolis, MN, United States)[20]. The sensitivity of detection was 38-1340 pg/mL.

Liver necroinflammation activity and fibrosis

Liver tissues were obtained by percutaneous transhepatic liver biopsy and routinely stained with hematoxylin and eosin (HE). The biopsies were examined by experienced pathologists. Hepatic necroinflammation activity and fibrosis stages were scored according to the METAVIR scoring system[23] as shown in Tables 1 and 2.

Liver immunohistochemistry

Biopsied hepatic tissues were immunohistochemically stained with anti-CXCL10 antibody (Abcam, Cambridge, United Kingdom). The staining intensity was assessed in 10 high-power fields based on the following scale: Score 0 – negative (-), staining absent; score 1 – weakly positive (+), yellowish; score 2 – moderately positive (++), brown; score 3 – strongly positive (+++), dark brown. The staining intensity in each field was calculated as: IS (intensity score) = Σ[(0 × F-)+(1 × F+)+(2 × F++)+(3 × F+++)], in which F is the percentage of cells stained at each intensity. The average score of 10 fields was the quantitative result of the whole slide[24]. Sections were scored by two independent observers.

Intrahepatic CXCL10 mRNA

RNA was extracted from biopsied liver tissue and subjected to quantitative real-time PCR using the following primers: Forward: 5′-CTGAATCCAGAATCGAAGGCCATC-3′; Reverse: 5′-TGTAGGGAAGTGATGGGAGAGG-3′. The expression was normalized to the expression of house-keeping gene β-actin using primers as described previously[20].

Antiviral therapy

Patients were treated with Peg-IFNα-2a (180 mg subcutaneous once weekly) plus RBV (1200 mg/d if body weight > 75 kg and 1000 mg/d if ⩽ 75 kg for HCV genotype 1; 800 mg/d for non-genotype 1)[21]. Genotype 1 OCI patients were treated for 48 wk and non-genotype 1 patients for 24 wk. For CHC patients, treatment was in accordance with the EASL guidelines[21]. Patients who received more than 80% of the cumulative total planned dose were considered to have completed the treatment[25]. All patients were followed up for 24 wk after treatment and a second liver biopsy was performed 24-96 wk after therapy in patients with informed consent.

Measures and monitoring

A standardized sample collection and data analysis protocol was applied at the five liver centers, including ELISA for serum anti-HCV antibodies (Roche Molecular Diagnostics, NJ, United States), highly sensitive, real-time PCR-based assay for HCV RNAs (LOD 15 IU/mL; COBAS Ampliprep/COBAS TaqMan 48 Analyzer, Roche), direct sequencing of serum or intrahepatic RNA for HCV genotyping (SinoMD, China), and Fibroscan for liver fibrosis (Echosens, France). Serum samples negative in routine HCV RNA tests were further ultracentrifuged and retested. If the ultracentrifuged serum remained HCV negative, PBMC were tested. In OCI patients, HCV RNA levels were re-assessed every 12 wk after initiation of antiviral treatment until the end of treatment or follow up. CHC patients had serum HCV RNA tests at 4 and 12 wk after initiation of treatment, and then every 12 wk until the end of treatment or follow up.

Statistical analysis

Allele frequencies for each SNP were determined by the Hardy-Weinberg equilibrium test and the differences between groups were examined by Chi-square tests. Qualitative results were expressed as frequency and percentage, and statistical analyses were performed using the Chi-square test or Fisher exact probability test. Quantitative data were expressed as mean ± SD and analyzed using the Student t-test. Stepwise binary logistic regression analysis was used to determine the influencing factors. A two-sided P value less than 0.05 was considered statistically significant, and odds ratios (ORs) and 95% confidence intervals (95%CI) were

Table 3 Clinical characteristics of patients enrolled in this study *n* (%)

	Seronegative OCI patients (*n* = 103)	Seropositive CHC patients (*n* = 155)	*P* value
Gender			0.664
Male	58 (56.3)	83 (53.5)	
Female	45 (43.7)	72 (46.5)	
Age (yr)	52.16 ± 7.64	42.70 ± 9.15	< 0.001
ALT (IU/L)	61.13 ± 23.54	93.17 ± 55.39	< 0.001
GGT	56.31 ± 16.63	52.86 ± 15.69	0.093
BMI	23.73 ± 2.38	24.12 ± 2.36	0.192
HOMA-IR	2.39 ± 0.15	2.42 ± 0.18	0.219
Transfusion/surgery/tattoo history	8 (7.8)	26 (16.8)	0.027
Family history of HCV infection	29 (28.2)	19 (12.3)	< 0.001
Intrahepatic HCV RNA (log_{10} IU/mL)	3.19 ± 1.05	5.48 ± 1.49	< 0.001
HCV genotype			0.89
Genotype 1	66 (64.1)	98 (63.2)	
Non-genotype 1	37 (35.9)	57 (36.8)	
Fibrosis (Fibroscan)			0.317
F0-1	65 (63.1)	104 (67.1)	
F2-4	38 (36.9)	51 (32.9)	
METAVIR activity score	1.14 ± 0.34	1.69 ± 0.68	< 0.001
METAVIR fibrosis score	1.82 ± 0.98	1.87 ± 1.07	0.673

P < 0.05; ALT: Alanine aminotransferase; GGT: Gamma-glutamyl transpeptidase; BMI: Body mass index; HOMA-IR: Homeostasis model assessment of insulin resistance; HCV non-genotype 1: Other HCV genotypes except for genotype 1, including HCV 2-6 genotypes.

assessed by SPSS software (v19.0; SPSS Inc., Chicago, IL, United States).

RESULTS

Clinical characteristics of seronegative OCI patients compared with seropositive CHC patients

The clinical characteristics of OCI and CHC patients are shown in Table 3. OCI patients were significantly older than CHC patients (52.16 years *vs* 42.70 years; *P* < 0.001) and had higher rates of HCV infection history in family members (28.2% *vs* 12.3%; *P* < 0.001). In contrast, the OCI group had lower base levels of ALT than the CHC group (61.13 ± 23.54 IU/L *vs* 93.17 ± 55.39 IU/L; *P* < 0.001), lower rates of blood transfusion/surgery/tattoo history (7.8% *vs* 16.8%; *P* = 0.027), lower levels of intrahepatic HCV RNA (3.19 ± 1.05 *vs* 5.48 log_{10} IU/mL ± 1.49 log_{10} IU/mL; P < 0.001), and lower METAVIR necroinflammation activity scores (1.14 ± 0.34 *vs* 1.69 ± 0.68; *P* < 0.001). No significant difference in HCV genotype and METAVIR fibrosis stages were observed between the two groups.

Serum CXCL10 levels in OCI patients with different polymorphisms of IL-28B rs12979860

Serum CXCL10 levels in OCI and CHC patients were compared. As shown in Figure 1, the OCI group exhibited significantly lower serum CXCL10 levels than the CHC group (192.91 ± 46.50 pg/mL *vs* 354.78 ± 102.91 pg/mL, *P* < 0.0001), irrespective of IL-28B rs12979860 C/C or non-C/C (C/T+ T/T). However, serum CXCL10 levels did not differ significantly between IL-28B rs12979860 polymorphism (OCI: 191.75 ± 45.04 pg/mL *vs* 211.67 ± 68.56 pg/mL, *P* = 0.311; CHC: 356.42 ± 106.10 pg/mL *vs* 347.0 ± 87.50 pg/mL, *P* = 0.667) (Figure 1).

CXCL10 polymorphisms in OCI patients compared with CHC patients

CXCL10 G-201A G/G genotype was more prevalent in the OCI group (93 of 103; 90.4%) than in the CHC group (116 out of 155; 74.8%; *P* = 0.008) (Table 4). The distribution of CXCL10 rs1440802 (C-1513T) did not differ significantly between the two groups (*P* = 0.733) (Table 4). Of the patients with IL-28B rs12979860 C/C genotype (Table 5), OCI was associated with a higher frequency of CXCL10 G-201A G/G (87 of 97; 89.7%) than CHC (95 of 128; 74.2%; OR = 0.33; 95%CI: 0.15-0.71; *P* = 0.005).

Serum and liver CXCL10 levels, and HCV RNA levels in OCI patients with different CXCL10 G-201A polymorphisms

The relationship between IL-28B rs12979860 polymorphism and CXCL10 is unclear. We showed that rs12979860 C/C patients had similar serum levels of CXCL10 to non-C/C patients in both the OCI and CHC group (Figure 1, *P* = 0.311 and 0.667). Due to the prevalence of IL-28B rs12979860 C/C genotype within both groups, we next compared the serum and liver CXCL10 levels and HCV RNA levels in rs12979860 C/C patients with different CXCL10 G-201A SNPs. Rs12979860 C/C OCI patients had lower serum CXCL10 levels, lower levels of intrahepatic CXCL10 mRNA (Figure 2B, 1.18 ± 0.27 *vs* 2.24 ± 0.65, *P* < 0.0001) and immunohistological staining scores (Figure 2C and D, 0.94 ± 0.34 *vs* 2.71 ± 0.52, *P* < 0.0001), as well as HCV RNA than CHC patients (Figure 2E; 3.20 ± 1.07 log_{10} IU/mL *vs* 5.53 ± 1.46 log_{10} IU/mL, *P* < 0.0001). In addition, within both groups, CXCL10 rs1439490 G/G patients had lower levels of serum and liver CXCL10 (Figure 2A, OCI: 184.82 ± 39.19 pg/mL *vs* 252.10 ± 49.52 pg/mL, *P* < 0.0001; CHC: 333.91 ±

Table 4 IL-28B single nucleotide polymorphism rs12979860 and CXCL10 single nucleotide polymorphism G-201A, C-1513T in occult hepatitis C virus infection and chronic hepatitis C virus infection patients *n* (%)

	Seronegative OCI patients (*n* = 103)	Seropositive CHC patients (*n* = 155)	*P* value
IL-28BSNP rs12979860			
C/C	97 (94.2%)	128 (82.6%)	0.009
Non-C/C	6 (5.8%)	27 (17.4%)	
CXCL10 SNP rs1439490 (G-201A)			
G/G	93 (90.4%)	116 (74.8%)	
G/A	9 (8.7%)	35 (22.6%)	0.003
A/A	1 (0.9%)	4 (2.6%)	
CXCL10 SNP rs1440802 (C-1513T)			
C/C	26 (25.2%)	40 (25.8%)	
C/T	54 (52.4%)	78 (50.3%)	0.733
T/T	23 (22.4%)	37 (23.9%)	

P value: OCI group compared with CHC group; $P < 0.05$; IL-28BSNP rs12979860 non-C/C genotype included IL-28BSNP rs12979860 C/T + T/T genotypes. OCI: Occult hepatitis C virus infection; CHC: Chronic hepatitis C virus infection.

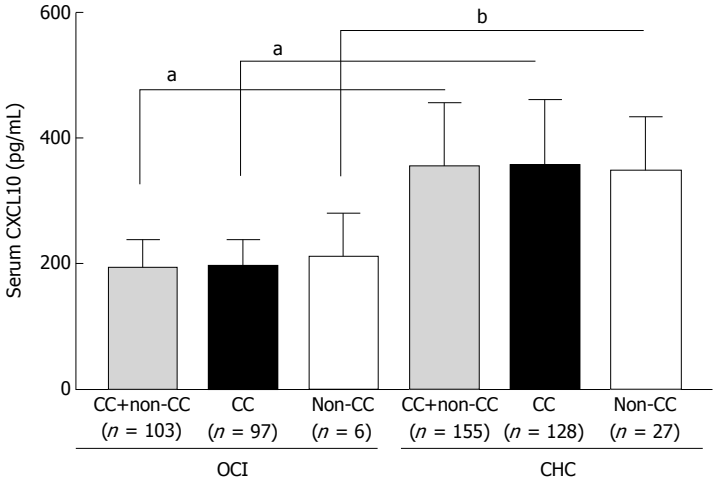

Figure 1 Serum CXCL10 levels in occult hepatitis C virus infection patients with different variants of IL-28B rs12979860 (C/C or non-C/C) as compared to chronic hepatitis C virus infection patients. [a]$P < 0.0001$; [b]$P = 0.001$. OCI: Occult hepatitis C virus infection; CHC: Chronic hepatitis C virus infection.

101.01 pg/mL *vs* 421.24 ± 94.19 pg/mL, $P < 0.0001$; Figure 2B, OCI: 1.16 ± 0.25 *vs* 1.42 ± 0.29, $P = 0.003$; CHC: 2.10 ± 0.61 *vs* 2.65 ± 0.60, $P < 0.0001$; Figure 2C and D, OCI: 0.91 ± 0.33 *vs* 1.20 ± 0.34, $P = 0.009$; CHC: 2.07 ± 0.51 *vs* 2.45 ± 0.47, $P < 0.0001$), except for intrahepatic HCV RNA (Figure 2E; OCI: 3.25 ± 1.09 $\log_{10}$ IU/mL *vs* 2.76 ± 0.66 $\log_{10}$ IU/mL, $P = 0.163$; CHC: 5.47 ± 1.42 $\log_{10}$ IU/mL *vs* 5.71 ± 1.58 $\log_{10}$ IU/mL, $P = 0.422$).

METAVIR scores in OCI patients with different variants of CXCL10 G-201A

In IL-28B rs12979860 C/C patients, those with OCI had lower hepatic necroinflammation scores than CHC patients, as evaluated by the METAVIR scoring system ($P < 0.0001$). However, METAVIR scored liver fibrosis stages did not differ significantly ($P = 0.67$). Necroinflammation activity (OCI: 1.05 ± 0.21 *vs* 1.80 ± 0.42, $P < 0.0001$; CHC: 1.60 ± 0.64 *vs* 2.09 ± 0.72, $P < 0.0001$) and fibrosis stage (OCI: 1.72 ± 0.99 *vs* 2.40 ± 0.69, $P = 0.04$; CHC: 1.64 ± 1.03 *vs* 2.58 ± 1.03, $P < 0.0001$) scores were both lower in CXCL10 G-201A G/G than in non-G/G SNP patients (Figure 3).

Efficacy of antiviral treatment in OCI patients with different CXCL10 G-201A variants

A total of 73 OCI patients and 90 CHC patients completed more than 80% of the planned antiviral drug doses. After 12 wk of treatment, ALT normalization occurred in 95.9% of OCI and 82.2% of CHC patients ($P < 0.05$; data not shown). In G-201A G/G OCI patients with lower baseline serum CXCL10, serum CXCL10 levels decreased to 60.90 ± 16.78 pg/mL and 57.2 5 ± 19.51 pg/mL at the endpoint of antiviral treatment and at 24 wk follow up, respectively. In G-201A G/G CHC patients, serum CXCL10 levels decreased to 89.77 ± 35.94 pg/mL and 73.33 ± 22.64 pg/mL at these time points - approximately 1.4-fold higher than in OCI patients (Figure 4).

Eight OCI patients who completed the course of treatment had a second liver biopsy. All these CXCL10 G-201A G/G patients had undetectable intrahepatic HCV RNA. CXCL10 mRNA, necroinflammation activity and fibrosis scores also decreased (Table 6). In contrast, of the 5 CHC patients who finished ⩾ 80% of planned doses and achieved SVR, 2 patients (1 G/G and 1 non-G/G) remained intrahepatic HCV RNA detectable

Table 5 CXCL10 single nucleotide polymorphism G-201A in occult hepatitis C virus infection and chronic hepatitis C virus infection patients with IL-28B rs12979860 C/C *n* (%)

	Seronegative OCI patients (*n* = 97)	Seropositive CHC patients (*n* = 128)	*P* value	OR (95%CI)
G/G	87 (89.7)	95 (74.2)	0.005	0.331
Non-G/G (G/A + A/A)	10 (10.3)	33(25.8)		(0.154-0.711)

P value: OCI group compared with CHC group; $P < 0.05$; OR: odds ratio; 95%CI: 95% confidence interval; CXCL10 rs1439490 non-G/G genotype included CXCL10 rs1439490 G/A + A/A genotypes. OCI: Occult hepatitis C virus infection; CHC: Chronic hepatitis C virus infection.

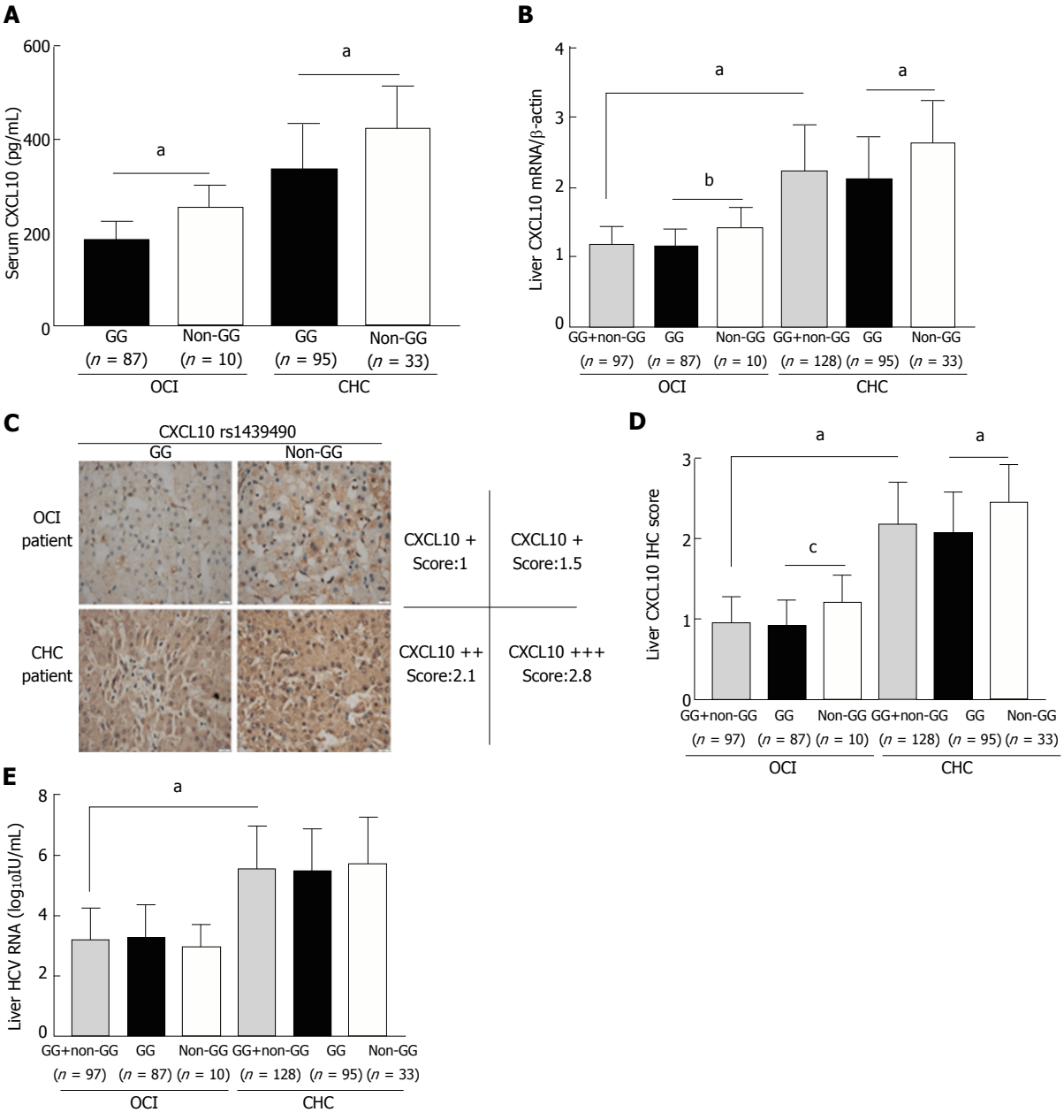

Figure 2 Comparison of serum CXCL10 levels (A), liver CXCL10 mRNA levels (B), representative (C) and grouped (D) liver CXCL10 IHC scoring, and liver hepatitis C virus RNA levels (E) among IL-28B rs12979860 C/C patients with different CXCL10 rs1439490 polymorphisms. [a]$P < 0.0001$; [b]$P = 0.003$; [c]$P = 0.009$. OCI: Occult hepatitis C virus infection; CHC: Chronic hepatitis C virus infection.

and only 3 patients achieved intrahepatic HCV RNA conversion to negative (2 patients with G-201A G/G and 1 patient with non-G/G). Nevertheless, CXCL10 mRNA, necroinflammation activity, and fibrosis scores in liver tissue all decreased after antiviral treatment (Table 7).

Logistic regression analysis of factors associated with OCI

As shown in Table 8, age, blood transfusion, family history of HCV infection, low levels of intrahepatic HCV RNA, IL-28B rs12979860 C/C genotype, and

Table 6 Intrahepatic hepatitis C virus RNA, CXCL10 mRNA, and METAVIR scores of 8 seronegative occult hepatitis C virus infection patients who underwent a second liver biopsy

Patient ID	CXCL10 G-201A SNP	Intrahepatic HCV RNA (log_{10} IU/mL)		Relative liver CXCL10 mRNA		METAVIR necroinflammation score		METAVIR fibrosis scores	
		Before	After	Before	After	Before	After	Before	After
1	G/G	2.17	(-)	1.20	0.80	1	0	0	0
2	G/G	3.57	(-)	0.95	0.90	1	0	1	1
3	G/G	2.72	(-)	0.74	0.63	1	1	1	1
4	G/G	5.08	(-)	1.31	0.94	1	0	1	0
5	G/G	2.83	(-)	1.42	0.64	1	0	0	0
6	G/G	1.97	(-)	1.06	0.83	2	1	1	1
7	G/G	4.64	(-)	1.03	0.70	2	0	2	1
8	G/G	2.94	(-)	1.26	0.71	1	1	3	1

SNP: Single nucleotide polymorphism; HCV: Hepatitis C virus.

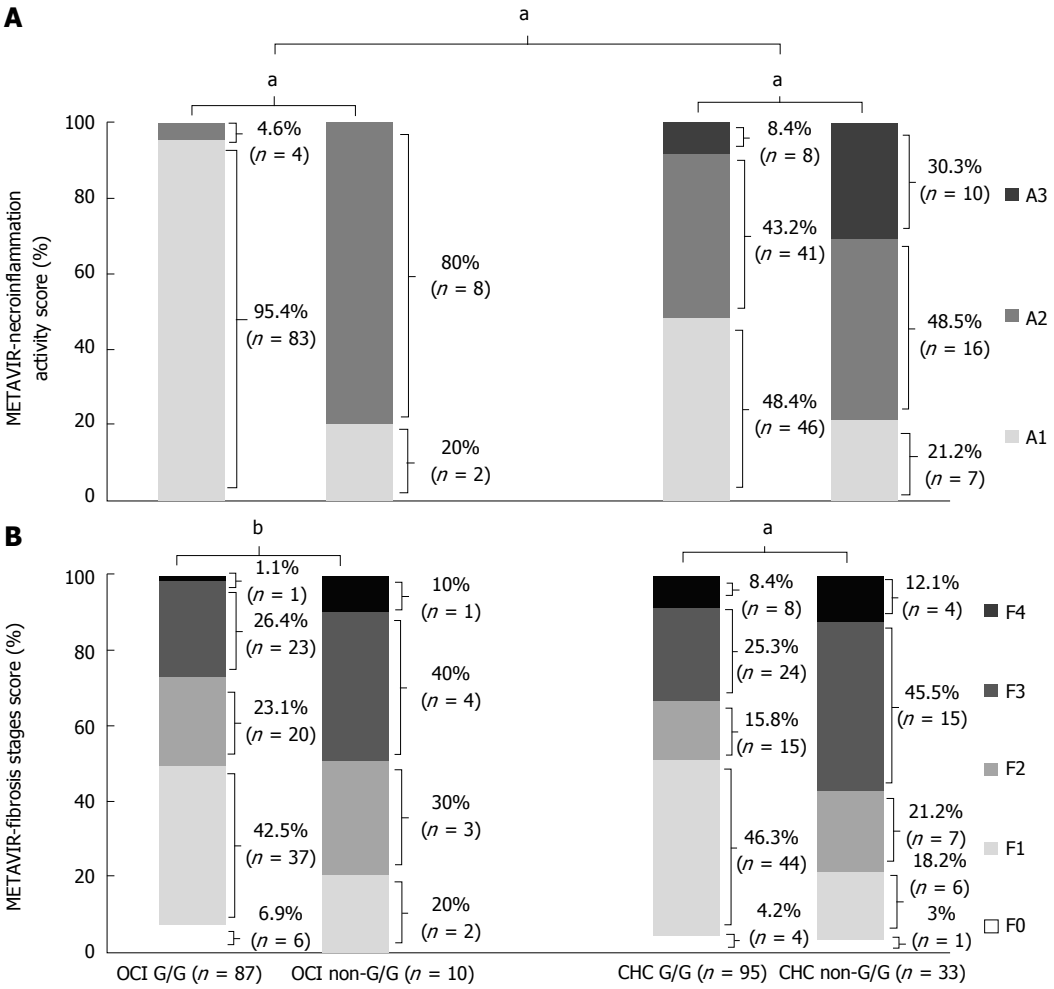

Figure 3 METAVIR necroinflammation activity (A) and fibrosis stage scores of patients with IL-28B rs12979860 CC genotype (B). [a]$P < 0.0001$; [b]$P = 0.04$. OCI: Occult hepatitis C virus infection; CHC: Chronic hepatitis C virus infection.

CXCL10 G-201A G/G genotype all influenced OCI occurrence (all $P < 0.05$). Furthermore, multivariate analysis showed that CXCL10 G-201A G/G genotype (OR = 0.31, 95%CI: 0.15-0.66; $P = 0.002$) and IL-28B rs12979860 C/C genotype (OR = 0.28, 95%CI: 0.11-0.71; $P = 0.008$) significantly influenced occult occurrence in patients with HCV infection.

DISCUSSION

CXC chemokine ligand (CXCL-10), also known as IFN-gamma inducible protein (IP-10), is a small and potent cytokine belonging to the C-X-C motif chemokine family. CXCL10 was previously considered an IFN-stimulated gene; however, CXCL10 induction

Table 7 Intrahepatic hepatitis C virus RNA, CXCL10 mRNA, and METAVIR scores of 5 seropositive chronic hepatitis C virus infection patients who underwent a second liver biopsy

Patient ID	CXCL10 G-201A SNP	Intrahepatic HCV RNA (log_{10} IU/mL)		Relative Liver CXCL10 mRNA		METAVIR necroinflammation score		METAVIR fibrosis scores	
		Before	After	Before	After	Before	After	Before	After
1	G/G	6.18	(-)	1.93	0.93	1	0	2	1
2	G/G	3.9	(-)	1.80	0.90	2	1	1	1
3	G/A	7.74	1.94	2.78	1.30	2	1	3	2
4	G/A	7.38	(-)	2.39	1.24	2	1	2	2
5	G/G	5.51	1.38	1.31	1.12	1	1	2	1

SNP: Single nucleotide polymorphism; HCV: Hepatitis C virus.

Table 8 Logistic regression analysis of factors associated with seronegative occult occurrence of hepatitis C virus

Variable	Univariate analysis			Multivariate analysis		
	OR	95%CI	*P* value	OR	95%CI	*P* value
Age	1.15	1.11-1.19	< 0.001			
Blood transfusion	0.39	0.17-0.92	0.031			
Family history of HCV	3.66	1.85-7.25	< 0.001			
Intrahepatic HCV RNA level	0.30	0.23-0.39	< 0.001			
IL-28B C/C	0.29	0.12-0.74	0.009	0.28	0.11-0.71	0.008
CXCL10 G-201A G/G	0.32	0.15-0.68	0.003	0.31	0.15-0.66	0.002

$P < 0.05$; OR: Odds ratio

in hepatocytes during acute HCV infection does not require IFNs. Infected hepatocytes and intrahepatic infiltrated lymphocytes secrete CXCL10 within the first days of HCV infection[26]. HCV-associated pathogen-associated molecular patterns (PAMPs) have recently been reported to be capable of directly activating the cellular innate immune pathways[26,27]. HCV RNAs or intermediates during viral replication can directly activate toll-like receptor 3 (TLR3) and retinoic acid-inducible gene-I (RIG-I), and induce the activation of nuclear factor-kappa B (NF-κB) *via* a myeloid differential protein-88-independent pathway (MyD88-independent pathway). NF-κB was found to positively regulate CXCL10 transcription during HCV infection as well as following exposure to poly(I·C) (a TLR3 agonist) and 5′ poly(U) HCV RNA (a RIG-I agonist) from two viral genotypes[26]. In addition, the transiently nuclear translocated interferon regulatory factor 3 (IRF3) was recruited to the proximal interferon sensitive response element (ISRE) during HCV infection and activated the CXCL10 promoter independently of type Ⅰ/Ⅲ IFN signaling. *In vitro* experiments also demonstrated that during early HCV infection, Huh7-derived cells expressing both TLR3 and RIG-I produced maximal CXCL10 mRNA with negligible induction of type Ⅰ or Ⅲ IFN, and neutralization of type Ⅰ and type Ⅲ IFN did not affect CXCL10 induction[26,27].

The engagement of CXCL10 with C-X-C motif chemokine receptor 3 (CXCR3) expressed on the surface of CD4+ T_h1 cells, natural killer (NK) cells, and CD8+ cytotoxic T cells induces the activation and migration of these cells to inflammatory sites[27,28]. Within the liver, the activated CD4+T_h1 cells produce more IFN-γ and tumor necrosis factor alpha (TNF-α), which in turn stimulate further secretion of CXCL10 from liver cells. Thus, the CXCL10-CXCR3 axis creates an amplification feedback loop (second paracrine signaling pathway) and maintains a sustained adaptive immune response, which plays an important role in viral suppression during acute HCV infection. However, this autoimmune process is not able to eliminate the virus in approximately 70% of patients and the virus persists for decades[29]. Instead, the CXCL10-CXCR3 signaling cytokines and cytotoxic factors released from CD4+ T_h1 cells contribute to chronic liver inflammation and is termed the CXCL10-mediated *non*-specific immune response.

This study and that by Bartolomé *et al*[19] revealed that serum CXCL10 levels in OCI patients were lower than in CHC patients, suggesting an underlying role of CXCL10 in the lower levels of HCV replication in OCI patients and the chronic immune response. IL-28B polymorphisms may affect the endogenous IFN-λ level, and thus are associated with low viral replication. We observed a higher prevalence of IL-28B rs12979860 C/C in OCI than in CHC patients, which is consistent with Bartolomé's report[19]. This phenomenon could partially explain the suppression of HCV replication in OCI patients; however, low expression of CXCL10 in the context of IL-28B C/C genotype-associated high endogenous IFN expression remains to be understood.

Deng *et al*[20] recently reported that two CXCL10 SNPs, G-201A and C-1513T, were overrepresented in Chinese populations from Beijing and Chongqing with chronic HBV infection. G-201A locates within the CXCL10 promoter region and is proximal to the NF-κB1/2 binding sites. G-201A SNP was associated with the expression of CXCL10 in PBMC and chronic

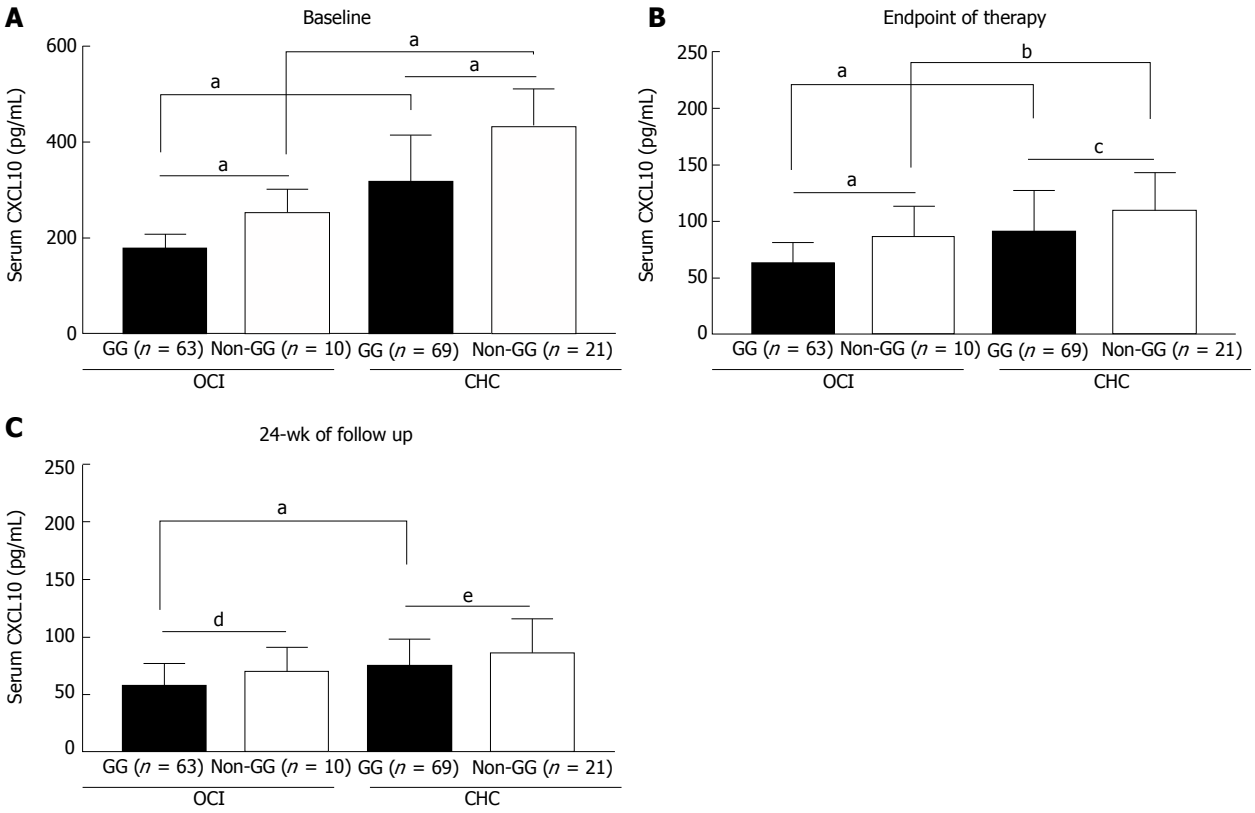

Figure 4 Effect of antiviral therapy on serum CXCL10 levels of IL-28 rs12979860 C/C patients with different CXCL10 G-201A single nucleotide polymorphisms. A: The baseline serum CXCL10 levels of IL-28 rs12979860 C/C patients who completed the Peg-IFNα plus ribavirin treatment; B-C: The serum CXCL10 levels of the patients at the endpoint of antiviral treatment (B) and at 24 wk follow up (C); [a]*P* < 0.0001; [b]*P* = 0.038; [c]*P* = 0.008; [d]*P* = 0.008; [e]*P* = 0.009. OCI: Occult hepatitis C virus infection; CHC: Chronic hepatitis C virus infection.

HBV disease progression[20]. This study inspired us to investigate whether these CXCL10 SNPs were associated with OCI. The general population in the Northeast area of China (Jilin and Heilongjiang Provinces) recruited in our study had a similar allele frequency of CXCL10 G-201A and C-1513T to the general population in Deng's study (data not shown). However, G-201A G/G genotype, but not C-1513T, was more prevalent in OCI patients. OCI patients with G-201A G/G had not only lower levels of serum CXCL10, but also lower levels of liver mRNA and CXCL10 protein. OCI patients also exhibited less severe liver METAVIR necroinflammation activity and fibrosis. We hypothesize that CXCL10 G-201A may influence the secretion of CXCL10, and subsequently the binding of CXCL10 to CXCR3 on the surface of T_h1 cells. As such, the CXCL10-CXCR3 axis-mediated adaptive immune response is compromised. This concession would affect spontaneous clearance of the virus, but may also cause less liver damage. These extremely low levels of HCV replication are not sufficient to elicit anti-HCV antibodies. However, long-term HCV replication still promotes liver disease.

With respect to antiviral treatment, the comprehensive antiviral efficacy was better in OCI patients than in CHC patients, with CXCL10 G-201A G/G OCI patients even better than non-G/G OCI patients. ALT normalization rate increased along with the decrease in serum CXCL10 level. Due to the requirement for intrahepatic biopsy, only 8 OCI patients and 5 CHC patients who finished antiviral treatment consented to receive a second liver biopsy after treatment. Nevertheless, the results showed a decreased tendency of METAVIR liver necroinflammation activity and fibrosis scores along with the decrease in liver CXCL10 mRNA and protein levels. All 8 OCI patients achieved conversion to liver HCV RNA negative and 5 CHC patients achieved seroconversion to HCV RNA negative with 3 cases of conversion to liver HCV RNA negative. The relationship between decreased serum CXCL10 levels in CHC patients and antiviral treatment efficacy is complicated. It remains to be determined whether this is due to the suppression of HCV RNA or direct inhibition of viral replication by CXCL10. High CXLC10 levels were considered to negatively affect the antiviral efficacy of IFN-based treatment. However, CXCL10 levels have also been reported to affect non-IFN therapy for HCV infection, and are implicated as a surrogate marker of intracellular viral replication complex decay[30,31].

In summary, our study revealed a higher prevalence of CXCL10 G-201A (rs1439490) G/G in OCI patients than in CHC patients. OCI patients with G-201A G/G achieved better antiviral efficacy with Peg-IFN plus RBV.

CXCL10 G-201A G/G is associated with a seronegative occult response to HCV infection, and may be an independent prognostic factor for IFN-based antiviral treatment. Our results suggest the potential clinical significance of CXCL10 G-201A genotyping in identifying OCI during chronic HCV infection. In addition, clarifying the correlation between CXCL0 rs1439490 and liver necroinflammation or fibrosis stage may also guide IFN-based antiviral treatment of CHC patients. However, given the current availability of direct acting antiviral agents, the relationship between CXCL10 G-201A G/G and IFN-free anti-HCV regimens requires further study.

ARTICLE HIGHLIGHTS

Research background

In the past two decades, some patients with chronic hepatitis C virus (HCV) infection (CHC) have been shown to be seronegative for anti-HCV antibodies and RNA, but have intrahepatic HCV RNA in liver biopsy. However, the etiology of this occult HCV infection (OCI) remains unclear.

Research motivation

Seronegative OCI patients were reported to have significantly lower serum CXCL10 levels than patients with CHC. Polymorphisms in the CXCL10 promoter have been implicated in the genetic variation underlying the susceptibility to chronic HBV infection (CHB) progression in Chinese populations. Moreover, CHC and CHB induce similar liver lesions and fibrosis through continuous infiltration of inflammatory cells, but do not damage hepatocytes directly. These phenomena promoted our interest to examine whether CXCL10 G-201A underlies the disease manifestation of OCI.

Research objectives

To investigate the allele frequency of CXCL10 single nucleotide polymorphisms (SNPs) in patients with OCI and whether they are associated with the low levels of CXCL10 in OCI patients.

Research methods

We characterized the expression frequency of CXCL10 G-201A (rs1439490), C-1513T (rs1440802), and IL-28B rs12979860 in seronegative OCI and seropositive CHC patients in Northeastern China. Serum CXCL10 levels were measured by ELISA. Intrahepatic CXCL10 levels were determined by quantitative PCR and immunohistochemical *semi*-quantitative scoring. Liver necroinflammation and fibrosis were scored according to the METAVIR system. The associations of CXCL10 rs1439490 with CXCL10 levels and antiviral efficacy in OCI were analyzed.

Research results

CXCL10 G-201A G/G was more prevalent in seronegative OCI patients than in seropositive CHC patients. Serum CXCL10 levels were lower in OCI patients than in CHC patients, but did not differ significantly between IL-28B rs12979860 C/C and non-C/C patients. Of IL-28B rs12979860 C/C patients, OCI patients with CXCL10 G-201A G/G had lower serum and liver levels of CXCL10, and lower levels of liver necroinflammation and fibrosis than non-G/G patients. OCI patients had high ALT normalization rates and serum CXCL10 decreased significantly after Peg-IFNα plus ribavirin treatment, most potently in G-201A G/G patients. Liver necroinflammation and fibrosis were alleviated in 8 OCI patients after treatment. Multivariate analysis indicated that CXCL10 G-201A G/G significantly influenced the occurrence of OCI in HCV infection.

Research conclusions

Our study revealed a higher prevalence of CXCL10 rs1439490 G/G genotype in OCI patients than in CHC patients. OCI patients with rs1439490 G/G genotype achieved better antiviral efficacy with Peg-IFN plus RBV. CXCL10 G-201A genotype is associated with the occurrence of seronegative OCI in patients with CHC, and may be *an independent prognostic factor for IFN-based antiviral treatment.*

Research perspectives

More paired liver biopsies before and after antiviral treatment are anticipated to examine the correlation of CXCL10 change with clinical outcomes of OCI. In addition, given the current availability of direct acting antiviral agents, the relationship between CXCL10 G-201A G/G and IFN-free anti-HCV regimens requires further study.

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META-ANALYSIS

Donor-to-recipient gender match in liver transplantation: A systematic review and meta-analysis

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Abstract

AIM

To perform a systematic review and meta-analysis on donor-to-recipient gender mismatch as a risk factor for post-transplant graft loss.

METHODS

A systematic literature search was performed using PubMed, Cochrane Library database and EMBASE. The primary outcome was graft loss after liver transplantation. Odds ratios and 95% confidence intervals were calculated to compare the pooled data between groups with different donor-to-recipient gender matches. Three analyses were done considering (1) gender mismatches (F-M and M-F) *vs* matches (M-M and F-F); (2) Female-to-Male mismatch *vs* other matches; and (3) Male-to-Female mismatch *vs* other matches.

RESULTS

A total of 7 articles were analysed. Gender mismatch (M-F and F-M) was associated with a significant increase of graft loss respect to match (M-M and F-F) (OR: 1.30; 95%CI: 1.13-1.50; $P < 0.001$). When F-M

mismatch was specifically investigated, it confirmed its detrimental role in terms of graft survival (OR: 1.83; 95%CI: 1.20-2.80; P = 0.005). M-F mismatch failed to present a significant role (OR: 1.09; 95%CI: 0.73-1.62; P = 0.68).

CONCLUSION

Gender mismatch is a risk factor for poor graft survival after liver transplantation. Female-to-male mismatch represents the worst combination. More studies are needed with the intent to better clarify the reasons for these results.

Key words: Graft survival; Female-to-male mismatch; Liver transplantation; Donor-to-recipient match; Gender

Core tip: Limited data exist on the role of donor-to-recipient gender mismatch after liver transplantation. This is the first systematic review and meta-analysis specifically investigating the role of gender match in the setting of liver transplant. Female-to-male mismatch was a risk factor for graft loss, with a 83-fold increased risk.

Lai Q, Giovanardi F, Melandro F, Larghi Laureiro Z, Merli M, Lattanzi B, Hassan R, Rossi M, Mennini G. Donor-to-recipient gender match in liver transplantation: A systematic review and meta-analysis. *World J Gastroenterol* 2018; 24(20): 2203-2210 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i20/2203.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i20.2203

INTRODUCTION

Liver transplantation (LT) represents the gold-standard therapy for the treatment of more than fifty liver disorders, consenting to obtain excellent results in terms of survival rates even in case of dreadful pathologies[1]. However, LT represents a scarce resource. As a consequence, a careful matching between donor and recipient should be done, with the main intent to optimize the results in terms of post-LT survivals[2]. Gender match seems to represent one of the aspects influencing outcomes after LT, although this association is largely controversial. Monocentric studies showed a correlation between donor gender and graft loss, mainly in case of female donor-to-male recipient (F-M) mismatch[3,4]. On the opposite, a large international study based on 16410 LT subjects did not find any correlation[5].

Recently, several scores aimed at identifying the quality of donors have been developed, with the main intent to optimize the donor-to-recipient matching and to predict post-transplant outcomes[6,7]. However, no one of them showed donor gender as a risk factor for poor graft survival, thus raising the question of whether donor-recipient gender mismatch truly impacts on survival rates.

The main aim of the present study is to report a systematic review of the literature and a meta-analysis focused on investigating the role of donor-to-recipient gender match in the setting of liver transplantation as a potential predictor of graft loss.

MATERIALS AND METHODS

Search strategy

A systematic search was done in relation to relevant studies focusing on the role of gender match in organ donation for LT. The search strategy was done in accordance with the Preferred Reporting Items for Systemic Reviews and Meta-Analysis (PRISMA) guidelines, as well as PRISMA for abstracts[8]. A search of the electronic databases MEDLINE-PubMed, Cochrane Library and EMBASE was conducted using the following research terms: (gender[tw] OR sex[tw]) AND (discordance[tw] OR mismatch[tw] OR match[tw]) AND (liver transplant*[tw]).

Text word [tw] was preferred respect to MeSH words with the intent to identify In Process citations. Studies published before March 15, 2018, were taken into consideration.

Screening process

The present qualitative systematic review included a priori search criteria of journal articles among adult (age $\geqslant$ 18 years) human patients. Studies were limited to the English language.

Exclusion criteria were: (1) Papers lacking sufficient statistical details; (2) review articles; (3) nonclinical studies; (4) expert opinions; (5) letters; (6) conference summaries; and (7) case reports.

Study selection

Two reviewers (QL and FG) independently screened the identified studies and their extracted data. In case of disagreement, the paper was discussed by all the authors.

Quality assessment

Selected studies were reviewed based on the representativeness of the study population, comparability of cohorts, adequate assessment of outcomes, sufficient length of follow-up, adequacy of follow-up, and source of study funding. The quality of the papers was assessed using the Newcastle-Ottawa Quality Assessment Scale (NOS): Studies with scores > 6 were defined as high-quality studies[9].

NOS details of each selected study were reported in Table 1. The characteristics coming from each study were collected in Table 2. The following features were collected: Author, year of publication, number of transplanted cases, investigated follow-up period of the study, number of cases for each donor-to-recipient gender combination (M-M, F-F, M-F, and F-M), graft

Table 1 Quality of studies evaluated by the modified Newcastle-Ottawa scale

Ref	Selection				Comparability		Outcome		Quality score
	Case definition	Representativeness	Selection of controls	Definition of controls	Comparable for therapy	Comparable for etiology	Assessment of outcomes	Integrity of follow-up	
Kahn *et al*[17]	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	★★★★★★★★
Marino *et al*[18]	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	★★★★★★★★
Grande *et al*[19]	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	★★★★★★★★
Berrevoet *et al*[20]	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	★★★★★★★★
Brooks *et al*[21]	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	★★★★★★★★
Croome *et al*[22]	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	★★★★★★★★
Grat *et al*[23]	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	★★★★★★★★

Table 2 Demographic and clinical aspects of the selected studies

Ref.	Year	n	FU (mo)	Number for group				Graft survival (%)				Patient survival (%)			
				M-M	F-F	M-F	F-M	M-M	F-F	M-F	F-M	M-M	F-F	M-F	F-M
Kahn *et al*[17]	1993	883	2	350	121	312	50	72	64	72	40	85	83	83	62
Marino *et al*[18]	1995	462	24	201	71	92	98	72	64	78	55	77	82	82	66
Grande *et al*[19]	1997	423	60	189	64	69	101	52	64	59	71	NA	NA	NA	NA
Berrevoet[20]	1997	105	6	40	12	32	21	65	67	66	71	78	100	81	86
Brooks *et al*[21]	1997	994	24	392	219	247	126	74	76	76	56	NA	NA	NA	NA
Croome *et al*[22]	2013	1042	120	412	217	249	164	75	65	76	59	NA	NA	NA	NA
Grat *et al*[23]	2015	76	120	-	29	47	-	-	75	73	-	NA	NA	NA	NA

FU: Follow-up; M: Male; F: Female; NA: Not available.

survival for each group reported at the last follow-up and patient survival for each group reported at the last follow-up.

Statistical analysis

Follow up period strongly varied from 2 to 120 mo in the investigated studies: graft survival rates were estimated at their last available value. Summary measures were extracted from each study and used to generate a pooled odds ratio (OR). Higgins I^2 statistic was used to assess heterogeneity. Higgins I^2 statistic values of 0-25%, 25%-50%, and > 50% were considered as indicative of homogeneity, moderate heterogeneity, and high heterogeneity, respectively. When Higgins I^2 statistic value was < 25%, a fixed-effects model was used. Conversely, if Higgins I^2 statistic value overpassed this threshold, a random-effects model was adopted. OR was considered statistically significant when the P value < 0.05; OR and 95%CI > 1 revealed a higher risk of graft loss, whereas a result < 1 had the opposite meaning. The analysis was performed using OpenMEE software (http://www.cebm.brown.edu/openmee/index.html).

RESULTS

The selection process of the articles is explained in Figure 1.

As for the selection process according to the PRISMA guidelines, the various examined databases provided a total of 137 articles to screen. Four more articles were added after manual research. After removal of 65 duplicates, 76 articles were available for the screening. According to the title and the abstract, 54 articles were removed. Of the remaining 22 papers, 15 were not considered eligible after full-text evaluation. Unfortunately, in 9 articles specifically investigating the role of gender matching in LT, not enough statistical information was available, thus determining their removal from further analyses[4-5,10-16].

Eventually, 7 articles were identified, with a total of 3935 investigated cases (Table 2)[17-23].

As for the quality of the reported studies, all the investigated articles were retrospective cohort studies all presenting the excellent NOS value of eight, thus reporting the overall high quality of the studies focused on this topic (Table 1).

Three studies were from European countries, three others were from the United States and one from Canada. Five of the reported studies were published before the year 2000. The number of reported cases ranged from 76 to 1042 subjects. Six studies reported all the possible combinations of gender match, while one study only reported M-F and F-F subjects[24-26]. Only looking at the six studies reporting all the possible combinations, M-M cases ranged from 38% to 45% of cases, F-F from 11% to 21%, M-F from 16% to 37% and F-M from 6% to 24%. Globally, M-M cases were 1584, F-F subjects were 743, M-F 1048 and F-M 560. Gender-matched cases (M-M and F-F) were 2327 (59%), whilst mismatched cases (M-F and F-M) were 1608 (41%).

Graft survival was reported in all the studies, although variable follow-up periods were used across the analysed series. In detail, M-M patients reported a graft survival ranging 52%-75%, F-F subjects 64%-75%, M-F

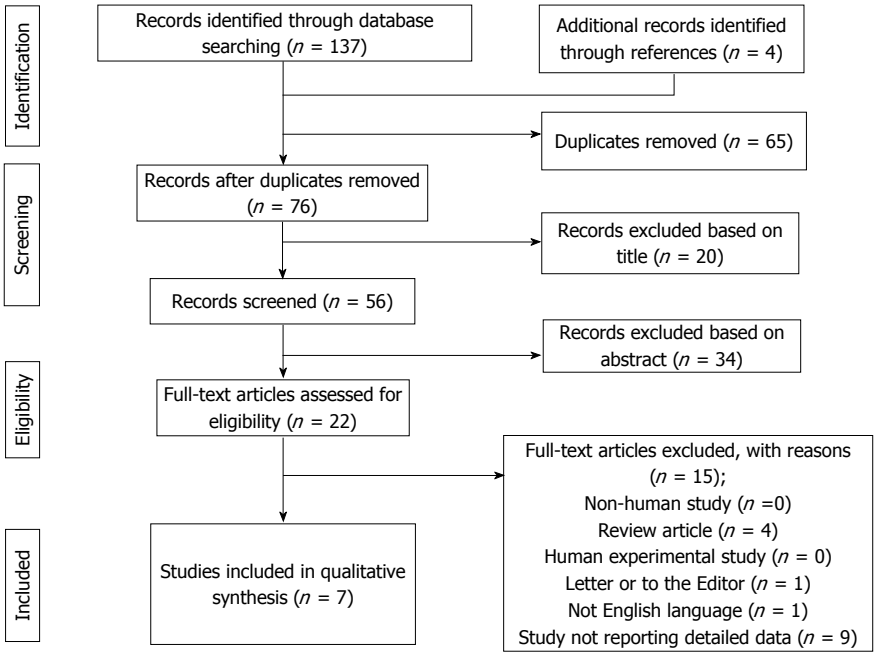


Figure 1 PRISMA flowchart of the literature search and study selection.

cases 59%-78% and F-M individuals 40%-71%.

Three different meta-analyses were performed. First, a fixed-effects model was realized comparing matched (M-M and F-F) and mismatched (F-M and M-F) cases. We observed a higher risk for graft loss in mismatched cases (OR: 1.13; 95%CI: 1.30-1.50; $P < 0.001$) (Figure 2A). Higgins I^2 statistic presented a value = 2.2% ($P = 0.41$), showing homogeneity among the examined studies; funnel plot also did not show publication biases (Figure 3A).

Then, starting from this evidence, two separated random-effects models were done investigating the specific role of F-M and M-F mismatches, respectively. When F-M mismatch was compared with the other three combinations, we reported a higher risk for graft loss in mismatched cases (OR: 1.83; 95%CI: 1.20-2.80; $P = 0.005$) (Figure 2B).

Higgins I^2 statistic presented a value = 75.8% ($P < 0.001$), showing a great heterogeneity among the examined studies; funnel plot showed the presence of publication biases (Figure 3B).

Lastly, when M-F mismatch was compared with the other three combinations, we did not report any increased risk for graft loss in mismatched cases (OR: 1.09; 95%CI: 0.73-1.62; $P = 0.68$) (Figure 2C). Higgins I^2 statistic presented a value = 80.5% ($P < 0.001$), showing a great heterogeneity among the examined studies; funnel plot showed the presence of publication biases (Figure 3B).

DISCUSSION

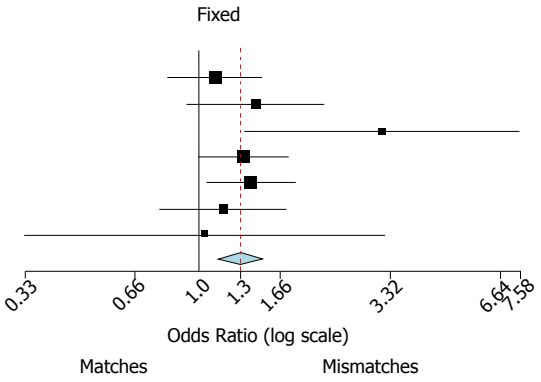
The results reported in the present meta-analysis suggest a detrimental role of the F-M mismatch in terms of graft survival. On the opposite, the M-F mismatch was not related to any negative course. These results may be connected with several possible explanations. For example, different donor female and male hormones should play a role in this phenomenon[11]. Some studies showed that a connection exists between estrogens and protection to ischemic injury: in other terms, when a female liver is removed from its homeostasis, the ischemic damage is major respect to a male one[24]. Estrogens also participate in favoring cholangiocyte proliferation and, consequently, the post-ischemic biliary repair[25].

Another possible explanation should be related to the differences in size among human females and males. Given that women are statistically smaller than men, and thus, by extension, have smaller livers, we should also postulate that an F-M mismatch may be connected with a greater risk for initial poor graft due to a small-for-size syndrome, a higher rate of complex vascular and biliary reconstruction due to the size discrepancy and, ultimately, longer warm ischemia times during the transplant[26]. Similar considerations should be done when other surrogates of size match have been investigated: for example, the American Donor Risk Index failed to demonstrate an effect of gender as a risk factor for graft failure, but the variable "height" was present, clearly demonstrating that a discrepancy in terms of donor-to-recipient size is an important risk factor[6].

It is interesting to note that the evidence that F-M mismatch is related to poor results has been reported in several experiences worldwide. A study from Japan showed that F-M mismatch related to a greater risk for patient death in a specific living-donor LT setting (OR: 2.10; 95%CI: 1.24-3.57; $P = 0.006$)[14]. A study from Germany based on 2144 LT cases showed that 1-,

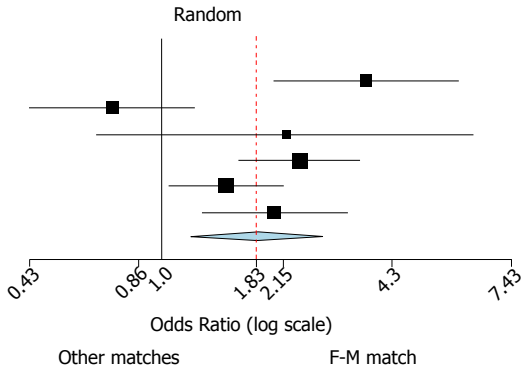
A

Studies	Estimate (95%CI)	Mismatches Ev/Trt	Matches Ev/Ctrl	Weights
Kahn	1.104 (0.821, 1.484)	116/257	246/576	22.8%
Grande	1.434 (0.931, 2.208)	54/116	116/307	10.9%
Berrevoet	3.187 (1.340, 7.581)	17/35	16/70	2.8%
Brooks	1.323 (0.996, 1.757)	116/274	257/720	24.7%
Croome	1.390 (1.044, 1.849)	116/254	297/788	24.4%
Marino	1.170 (0.785, 1.742)	63/144	127/318	12.8%
Grat	1.036 (0.332, 3.233)	10/16	37/60	1.6%
Overall (I^2 = 2.23%, P = 0.408)	1.302 (1.127, 1.504)	492/1096	1096/2839	100%



B

Studies	Estimate (95%CI)	F-M match Ev/Trt	Other matches Ev/Ctrl	Weights
Kahn	3.674 (2.044, 6.605)	30/257	20/576	16.3%
Grande	0.726 (0.430, 1.226)	23/116	78/307	17.4%
Berrevoet	2.207 (0.656, 7.428)	6/35	6/70	8.0%
Brooks	2.385 (1.626, 3.500)	56/274	70/720	19.8%
Croome	1.501 (1.041, 2.164)	51/254	113/788	20.1%
Marino	2.036 (1.285, 3.226)	43/144	55/318	18.5%
Overall (I^2 = 75.76%, P < 0.001)	1.830 (1.199, 2.795)	209/1080	342/2779	100%



C

Studies	Estimate (95%CI)	M-F match Ev/Trt	Other matches Ev/Ctrl	Weights
Kahn	0.779 (0.572, 1.060)	86/257	226/576	19.7%
Grande	2.582 (1.515, 4.401)	31/116	38/307	16.0%
Berrevoet	2.750 (1.034, 7.316)	11/35	10/70	9.6%
Brooks	0.779 (0.574, 1.113)	60/274	187/720	19.4%
Croome	1.129 (0.814, 1.565)	65/254	184/788	19.4%
Marino	0.551 (0.321, 0.946)	20/144	72/318	15.9%
Overall (I^2 = 80.46%, P < 0.001)	1.088 (0.729, 1.624)	273/1080	717/2779	100%

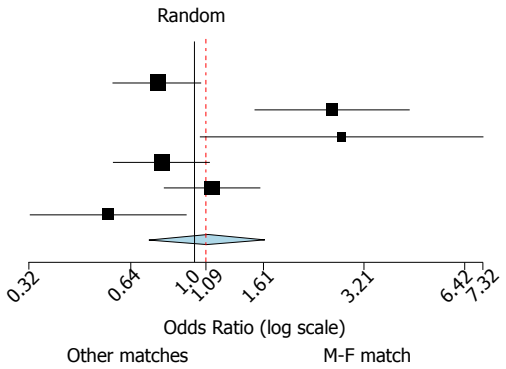


Figure 2 Forest plot result. A: Forest plot of odds ratios and 95% confidence intervals for the association between any donor-to-recipient mismatch (F-M and M-F) and graft survival in patients undergoing liver transplantation. Weights are from binary fixed-effect analysis; B: Forest plot of odds ratios and 95% confidence intervals for the association between donor-to-recipient F-M mismatch and graft survival in patients undergoing liver transplantation. Weights are from binary random-effect analysis; C: Forest plot of odds ratios and 95% confidence intervals for the association between donor-to-recipient M-F mismatch and graft survival in patients undergoing liver transplantation. Weights are from binary random-effect analysis.

5-, 10- and 15-year graft survival rates progressively decremented starting from the M-F combination (84%, 76%, 68% and 61%) to the F-F match (83%, 76%, 64% and 56%), the M-M match (85%, 72%, 63% and 53%) and, lastly, the F-M mismatch (80%, 66%, 56% and 49%) (P = 0.003)[15].

However, some caution should be taken into account in definitively considering F-M mismatch as a risk factor for graft loss. It is, in fact, important to underline that several confounders should influence the results observed in our study. An interesting study from the United States investigated a large multicentric population of 28222 LT recipients, showing that female donors were different respect to male ones for several risk factors of poor post-LT course, like age (median: 47 years *vs* 39 years), height (165 cm *vs* 178 cm), and cerebrovascular accident as cause of death (59% *vs* 35%) (P < 0.001): F-M mismatch was associated with a 17% increased risk of graft loss respect to an M-M match (95%CI: 1.11-1.24; P < 0.001), whereas M-F mismatch was not (HR = 1.02; 95%CI: 0.96-1.09; P = 0.46)[12]. These results are absolutely in line with the results observed in the present meta-analysis. However, when F-M mismatch was adjusted for significant recipient- and donor-related risk factors, its association with graft loss disappeared (HR = 0.95; 95%CI: 0.89-1.02; P = 0.18)[12].

The present study presents some shortcomings.

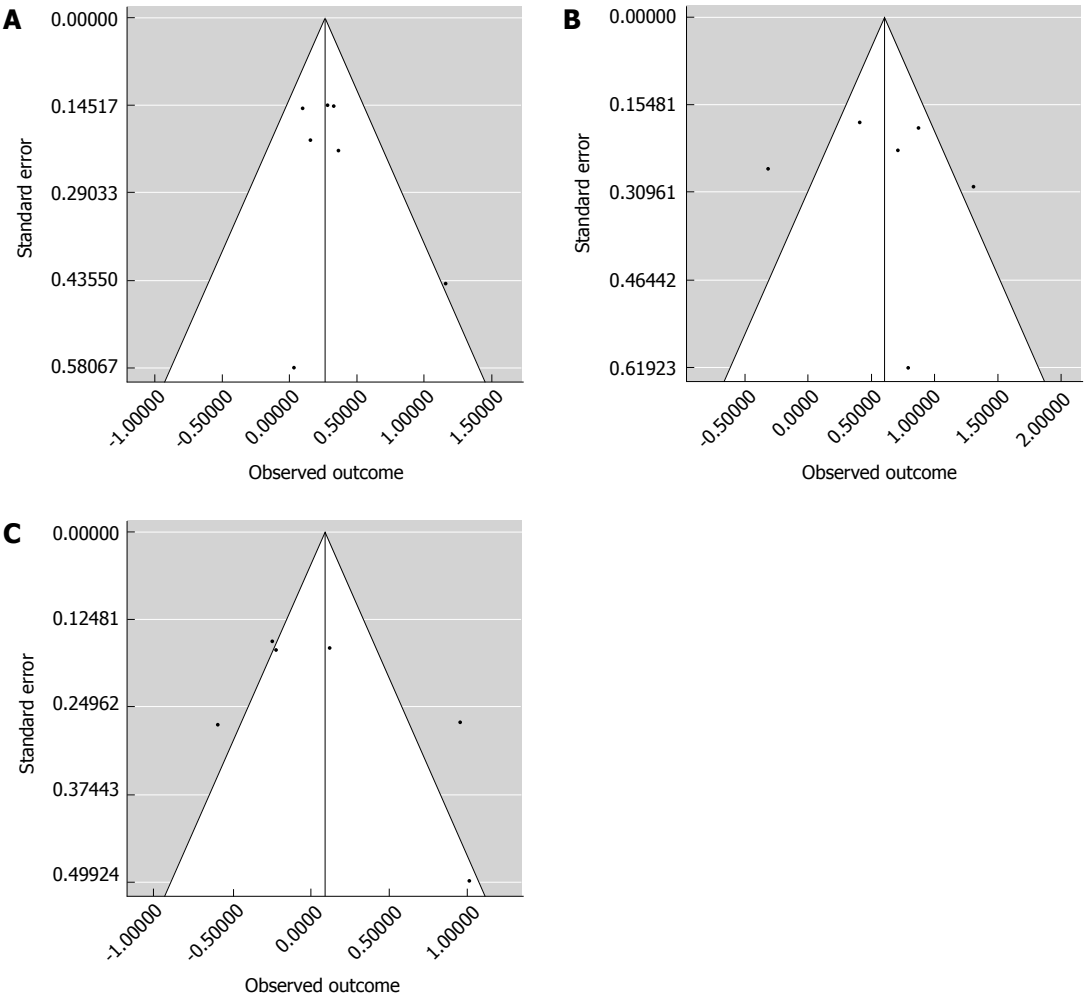


Figure 3 Funnel plots of the patients undergoing liver transplantation. A: Funnel plot of the seven studies investigating the association between any donor-to-recipient mismatch (F-M and M-F) and graft survival in patients undergoing liver transplantation; B: Funnel plot of the six studies investigating the association between donor-to-recipient F-M mismatch and graft survival in patients undergoing liver transplantation; C: Funnel plot of the six studies investigating the association between donor-to-recipient M-F mismatch and graft survival in patients undergoing liver transplantation.

The observed results should suggest the necessity of a meta-regression for minimizing the effect of potential confounders (donor age, donor ethnicity, ischemia time duration, and the presence of donor co-morbidities). Unfortunately, it was impossible to perform such an analysis according to the data obtainable from the selected studies. Funnel plots confirmed the presence of study biases, further suggesting the idea that some confounders may participate in altering the results of the meta-analysis. Another possible shortcoming of the present study is connected with the great heterogeneity observed among the studies in terms of the follow-up period. We can only assume that, although some studies presented a very short period of observation (only 60 d in one case[17]), such a period was able to capture a significant number of events: it is, in fact, clear that the first post-LT months typically represent the period in which the higher rate of graft loss is observed. Lastly, some studies were performed in the early nineties, thus reporting the early results of some LT centers. However, we should report that the negative role of F-M mismatch was observed also in more recent studies[22,23].

In summary, female to male donor-recipient mismatch represents a risk factor for graft loss after liver transplantation, with an 83-fold increased risk of graft failure. Several mechanisms should be postulated: Hormones, a major vulnerability to ischemic damages or size discrepancies have been advanced as possible explanations. However, some confounders should be taken into account. As a consequence, further large studies trying to design well-calibrated studies are needed, with the intent to definitively clarify the potential detrimental role of gender mismatch in the setting of liver transplantation.

ARTICLE HIGHLIGHTS

Research background

Donor-to-recipient gender match has been described as a possible risk factor for post-liver transplant outcomes, mainly when a female-to-male mismatch is done. However, no definitive data exist on this aspect, with only some, mainly monocentric, studies showing somewhat contrasting results. The impact of a meta-analysis on this aspect should be great, mainly in function of the opportunity to clarify a capital element of the organ allocation process in the

setting of liver transplantation.

Research motivation

The main aim of the present study is to clarify the role of donor-to-recipient gender mismatching in the setting of liver transplantation. The problem connected to this research is that no definitive clarity exists on the possible risks connected with the use of female donors for transplanting male recipients, although several studies raised on some concerns about this specific matching. The possibility to better clarify this aspect is connected with a safer opportunity to allocate organ during liver transplantation, thus improving the postoperative outcomes of subjects undergoing this type of transplant.

Research objectives

The main objective of the study was to better clarify the role of donor-to-recipient gender mismatch as a possible real risk factor for post-liver transplant graft and patient survival, or if its negative role was caused by several other confounding aspects in the allocation process.

Research methods

Three separate meta-analyses were realized after the systematic collection of all the articles available on English literature focused on the specific argument of donor-to-recipient gender match. First, a meta-analysis focused on the comparison between matched and mismatched cases was done. After this, two separate analyses were done specifically looking at the F-M and M-F mismatches.

Research results

According to the observed results, donor-to-recipient gender mismatch represented a risk factor for post-transplant outcomes, with a 30-fold increased risk for graft loss. When F-M mismatch was specifically investigated, an 83-fold increased risk for graft loss was reported, while such a risk was not present when an M-F mismatch was investigated. Despite the results confirmed the negative role of an F-M mismatch, open questions remained on its effective role, mainly in light of the presence of possible confounding factors potentially justifying these poorer results *(i.e.*, donor and recipient age, recipient disease severity and cause, donor ethnicity, ischemia time duration, and the presence of donor co-morbidities).

Research conclusions

Gender mismatch is a risk factor for poor graft survival after liver transplantation. Female-to-male mismatch represents the worst combination. A particular caution should be taken into account when this combination is present, thus improving the elements to consider during the organ allocation process.

Research perspectives

New studies are needed in this specific setting, with the intent to better clarify the reasons for the poor graft survivals observed in presence of a donor-to-recipient F-M gender mismatch. These studies mainly need to explore the possible confounders potentially being the cause for the reported results.

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