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TOPIC HIGHLIGHT

2015 Advances in Gastric Cancer

Anticancer effect of adenosine on gastric cancer *via* diverse signaling pathways

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Abstract

Extracellular adenosine induces apoptosis in a variety of cancer cells *via* intrinsic and extrinsic pathways. In the former pathway, adenosine uptake into cells triggers apoptosis, and in the latter pathway, adenosine receptors mediate apoptosis. Extracellular adenosine also induces apoptosis of gastric cancer cells. Extracellular adenosine is transported into cells through an adenosine transporter and converted to AMP by adenosine kinase. In turn, AMP activates AMP-activated protein kinase (AMPK). AMPK is the factor responsible for caspase-independent apoptosis of GT3-TKB gastric cancer cells. Extracellular adenosine, on the other hand, induces caspase-dependent apoptosis of MKN28 and MKN45 gastric cancer cells by two mechanisms. Firstly, AMP, converted from intracellularly transported adenosine, initiates apoptosis, regardless of AMPK. Secondly, the A_3 adenosine receptor, linked to G_i/G_q proteins, mediates apoptosis by activating the G_q protein effector, phospholipase Cγ, to produce inositol 1,4,5-trisphosphate and diacylglycerol, which activate protein kinase C. Consequently, the mechanisms underlying adenosine-induced apoptosis vary, depending upon gastric cancer cell types. Understand the contribution of each downstream target molecule of adenosine to apoptosis induction may aid the establishment of tailor-made chemotherapy for gastric cancer.

Key words: Adenosine; Apoptosis; Intrinsic pathway; Extrinsic pathway; Gastric cancer

Core tip: Emerging evidence has pointed to adenosine as a tumor suppressor. The most crucial problem for chemotherapy is side effects. Adenosine is an endogenous substance, and therefore, no or fewer side effects are expected for chemotherapy using adenosine. Extracellular adenosine induces apoptosis of gastric cancer cells through intrinsic and extrinsic signaling pathways. Adenosine and its signaling cascades, therefore, could represent a promising drug for gastric cancer chemotherapy. Moreover, the contribution of each downstream target molecule of adenosine to apoptosis induction may aid the establishment of tailor-made chemotherapy for gastric cancer.

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INTRODUCTION

Adenosine is an endogenous purine nucleoside composed of an adenine attached to a ribose sugar molecule moiety, and is present ubiquitously in all the organs, tissues and cells. One of the major roles of adenosine is as an energy supplier by conversion to adenosine diphosphate (ADP) and adenosine triphosphate (ATP). The adenosine concentration in organs and tissues is approximately 300 nmol/L under the normal conditions, but elevates to 600-1200 nmol/L under the inflammatory or ischemic conditions, where adenosine exhibits a protective effect against inflammatory or ischemic damage. Moreover, adenosine is implicated in a wide-range of signal transduction pathways relevant to cell proliferation and differentiation, cellular metabolism, apoptosis and cognitive function.

Accumulating evidence has shown that adenosine induces apoptosis in a variety of cancer cells *via* an intrinsic pathway linked to adenosine uptake into cells and the ensuing signaling cascades; its also functions through an extrinsic pathway linked to adenosine receptors[1,2]. The present study focused upon the antitumor effect of adenosine on gastric cancer cells and discussed the underlying mechanism.

ADENOSINE-INDUCED APOPTOSIS THROUGH THE EXTRINSIC PATHWAY

Adenosine receptors are coupled to G-proteins and are classified into A_1, A_{2a}, A_{2b} and A_3 receptors[3,4]. A_1, A_{2a} and A_{2b} adenosine receptors are well conserved during evolution and are highly homologous; however, the A_3 adenosine receptor varies, depending upon species[5]. A_1, A_{2a} and A_3 adenosine receptors are activated by physiological concentrations of adenosine (10-100 nmol/L), while the A_{2b} adenosine receptor is activated by much higher concentrations (over 1 μmol/L).

The A_1 adenosine receptor is linked to G_i/G_o proteins, causing inhibition of the G_i protein effector adenylate cyclase (AC), to reduce cAMP production and inhibit protein kinase A (PKA). Activation of the G_o protein effector phospholipase C-β (PLCβ) produces inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG) followed by activation of protein kinase C (PKC) (Figure 1)[6,7]. The A_1 adenosine receptor mediates apoptosis of CW2 human colon cancer cells and RCR-1 astrocytoma cells by activating caspase-3, -8, and -9[8,9]. In addition, evidence implicates the A_1 adenosine receptor in the apoptosis of breast cancer cells and gastric cancer cells[10,11].

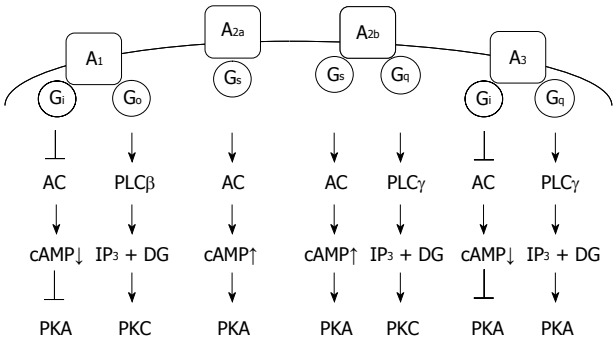

Figure 1 Adenosine receptors and their relevant signaling pathways. A_1: A_1 adenosine receptor; A_{2a}: A_{2a} adenosine receptor; A_{2b}: A_{2b} adenosine receptor; A_3: A_3 adenosine receptor; PLC: Phospholipase C; IP3: Inositol 1,4,5-trisphosphate; DG: Diacylglycerol; PKA: Protein kinase A; PKC; Protein kinase C.

The A_{2a} adenosine receptor is linked to the G_s protein, which activates the effector AC, to produce cAMP and activate PKA (Figure 1). The A_{2a} adenosine receptor, expressed in the striatum, is linked to the G_{olf} protein, causing AC activation, similar to G_s protein[12]. The A_{2a} adenosine receptor mediates apoptosis of Caco-2 human colon cancer cells by activating caspase-3/-9[13], and HepG2 human hepatoma cells by downregulating expression of Bcl-X_L and upregulating expression of Bid[14].

The A_{2b} adenosine receptor is linked to G_s/G_q proteins, causing the activation of the G_s protein effector AC, to produce cAMP and activate PKA, and activation of the G_q protein effector PLCγ, to produce IP_3 and DG followed by activation of PKC (Figure 1). The A_{2b} adenosine receptor mediates apoptosis of ovarian cancer cells by downregulating Bcl-2, upregulating Bax and activating caspase-3[15]. The A_{2b} adenosine receptor signaling, stimulated in a p73-dependent manner, enhances the apoptosis of a variety of cancer cells[16]. Conversely, blockage of the A_{2b} adenosine receptor inhibited the growth of prostate cancer cells[17]. This suggested that A_{2b} adenosine receptor promotes growth of prostate cancer cells.

The A_3 adenosine receptor is linked to G_i/G_q proteins, causing inhibition of the G_i protein effector AC, to reduce cAMP production and inhibit PKA, and activation of the G_q protein effector PLCγ, to produce IP_3 and DG, followed by activation of PKC (Figure 1)[18,19]. A recent review highlighted the A_3 adenosine receptor as a new target for cancer therapy. Chloro-N6-(3-iodobenzyl)-adenosine-5′-*N*-methyl-uronamide (Cl-IB-MECA), an agonist of the A_3 adenosine receptor, arrests the cell cycle at the G_0/G_1 phase and induces apoptosis of lung cancer cells by downregulating cyclin D1, c-myc, and CDK4, activating caspase-3 and -9, cleaving poly(ADP-ribose) polymerase, and inhibiting Akt[20]. Alternatively, Cl-IB-MECA suppresses proliferation and induces apoptosis of bladder cancer

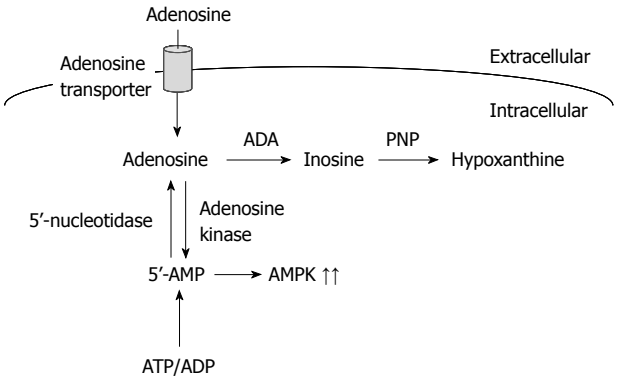

Figure 2 Intracellularly transported adenosine and the ensuing signaling pathways. AMPK: AMP-activated protein kinase; ADP: Adenosine diphosphate; ATP: Adenosine triphosphate.

cells through an extracellular signal-regulated kinase/c-Jun N-terminal kinase pathway[21]. CF102, another agonist of the A_3 adenosine receptor, induces apoptosis of hepatocellular carcinoma cells by de-regulating Wnt/NF-κB signal transduction pathways[22]. Overexpression of the A_3 adenosine receptor suppresses proliferation and induces apoptosis of malignant mesothelioma cells by inhibiting an Akt/NF-κB signaling pathway[23]. Overall, the A_3 adenosine receptor appears to suppress proliferation and induce apoptosis of cancer cells through diverse signaling pathways.

ADENOSINE-INDUCED APOPTOSIS THROUGH AN INTRINSIC PATHWAY

Adenosine also induces apoptosis of cancer cells through an intrinsic pathway. Extracellular adenosine is taken up into cells through adenosine transporters and converted to AMP by adenosine kinase, and AMP activates AMP-activated protein kinase (AMPK) (Figure 2)[24,25]. Adenosine, on the other hand, is metabolized into inosine by adenosine deaminase. In turn, inosine is metabolized into hypoxanthine by purine nucleoside phosphorylase (Figure 2). AMP derived from intracellularly transported adenosine upregulates p53 expression, to induce caspase-independent apoptosis of malignant pleural mesothelioma cells[26]. AMP also induces apoptosis of HuH-7 human hepatoma cells by downregulating c-FLIP expression, which is responsible for caspase-8 activation, followed by activation of the effector caspase-3[27]. Alternatively, intracellularly transported adenosine upregulates the expression of DIABLO and stimulates DIABLO release from the mitochondria, which neutralizes the inhibition of caspase-3 caused by inhibitor of apoptosis protein (IAP), together with downregulation of IAP2 expression, which leads to activation of caspase-3, to induce apoptosis of HuH-7 cells[28]. AMPK, activated by intracellularly transported adenosine and the ensuing conversion to AMP, phosphorylates Bcl-X_L, causing disruption of mitochondrial membrane potential and stimulation of DIABLO release from the mitochondria in HuH-7 cells[29]. Furthermore, intracellularly transported adenosine, but not AMP or AMPK, induces apoptosis of MCF-7 human breast cancer cells by accumulating AMID in the nucleus in a caspase-independent manner[30].

ADENOSINE-INDUCED APOPTOSIS OF GASTRIC CANCER CELLS

We have found that extracellular adenosine induces apoptosis of the gastric cancer cell lines GT3-TKB[31], MKN28 and MKN45 cells (unpublished data). Adenosine-induced GT3-TKB cell death was significantly inhibited by an inhibitor of the adenosine transporter or an inhibitor of adenosine kinase, while it was not affected by inhibitors of adenosine receptors[31]. This suggested that adenosine transporter-mediated uptake into cells, and adenosine kinase-mediated conversion to AMP, are required for extracellular adenosine-induced apoptosis of GT3-TKB cells. AMPK is activated in response to a cytosolic AMP rise, and therefore, AMPK may execute apoptosis of GT3-TKB cells as a downstream target of AMP (Figure 3A). Extracellular adenosine had no effect on mitochondrial membrane potentials in GT3-TKB cells and adenosine-induced apoptosis was not inhibited by caspase inhibitors[31]. This indicated that extracellular adenosine induces caspase-independent apoptosis of GT3-TKB cells apoptosis through an intrinsic pathway.

A study showed that extracellular adenosine causes cell cycle arrest and induces apoptosis of HGC-27 human gastric cancer cells[32]. Adenosine-induced HGC-27 cell apoptosis was inhibited by an inhibitor of the adenosine transporter; however, it was not affected by a broad inhibitor of P_1 receptors or a non-selective antagonist of P_2 receptors. This supported the involvement of the intrinsic pathway in adenosine-induced apoptosis of gastric cancer cells (Figure 3A).

Extracellular adenosine-induced apoptosis of MKN28 and MKN45 cells was inhibited by an inhibitor of adenosine transporter or an inhibitor of adenosine kinase, although an AMPK activator did not induce apoptosis. This suggested that the intrinsic pathway participates in adenosine-induced apoptosis of MKN28 and MKN45 cells; in other words, intracellularly transported adenosine and converted AMP trigger apoptosis of MKN28 and MKN45 cells (Figure 3B).

Extracellular adenosine-induced apoptosis of MKN28 and MKN45 cells was also inhibited by an inhibitor of the A_3 adenosine receptor. This implied that the extrinsic pathway also participates in adenosine-induced apoptosis of MKN28 and MKN45 cells. The A_3 adenosine receptor is linked to G_i/G_q proteins involving PKA inhibition and PKC activation[18,19]. Adenosine-induced MKN28 cell apoptosis was abolished by a PKC inhibitor. This indicated that the G_q protein linked to the A_3

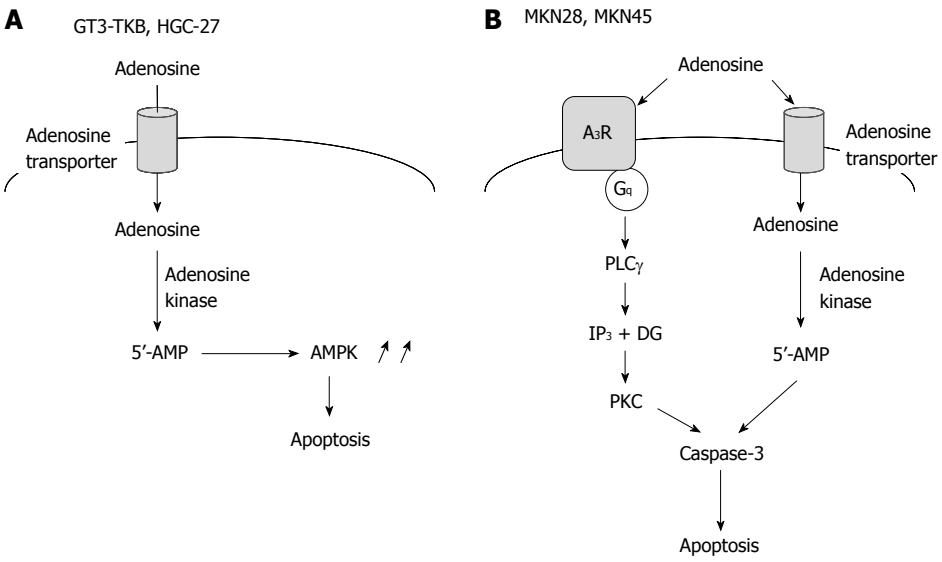

Figure 3 Extracellular adenosine-induced apoptosis of gastric cancer cells. A: The adenosine-induced apoptotic pathway for GT3-TKB and HGC-27 cells; B: The adenosine-induced apoptotic pathway for MKN28 and MKN45 cells. AMPK: AMP-activated protein kinase.

adenosine receptor is responsible for adenosine-induced apoptosis of MKN28 cells (Figure 3B). Adenosine activated only caspase-3 in both in MKN28 and MKN45 cells, but caspase-4, -8 and -9 were not affected. This suggested that adenosine-induced caspase-3 activation is independent of endoplasmic reticulum (ER) stress relevant to caspase-4 activation, death receptors relevant to caspase-8 activation or oxidative stress relevant to mitochondrial damage and caspase-9 activation. How extracellular adenosine's activation of caspase-3 in MKN28 and MKN45 cells remains to be explored.

Extracellular adenosine exhibits a beneficial antitumor effect on a variety of cancer cells including gastric cancer cells. Adenosine is an endogenous substance, and therefore, no or fewer side effects are expected for chemotherapy using adenosine. Thus, adenosine could be developed as a promising drug for gastric cancer chemotherapy. Moreover, the ability of downstream target molecules of adenosine to induce apoptosis of individual gastric cancer cell types, might make them targets for tailor-made chemotherapy for gastric cancer. Especially, specific potential inhibitors and targets of the A_3 adenosine receptor and AMP, respectively, could be used therapeutically.

CONCLUSION

Adenosine has the potential to induce apoptosis of gastric cancer cells through diverse intrinsic and extrinsic signaling pathways. The underlying mechanisms vary, depending upon gastric cancer cell types. Understanding the contribution of each downstream target molecule of adenosine to the induction of gastric cancer cell apoptosis, therefore, may aid the establishment of tailor-made chemotherapy for gastric cancer.

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TOPIC HIGHLIGHT

2015 Advances in Gastric Cancer

Evaluation and treatment of malignant ascites secondary to gastric cancer

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Abstract

Malignant ascites affects approximately 10% of patients with gastric cancer (GC), and poses significant difficulties for both patients and clinicians. In addition to the dismal general condition of affected patients and the diversity of associated complications such as jaundice and ileus, problems in assessing scattered tumors have hampered the expansion of clinical trials for this condition. However, the accumulation of reported studies is starting to indicate that the weak response to treatment in GC patients with malignant ascites is more relevant to their poor prognosis rather than to the ascites volume at diagnosis. Therefore, precise assessment of initial state of ascites, repetitive evaluation of treatment efficacy, selection of suitable treatment, and swift transition to other treatment options as needed are paramount to maximizing patient benefit. Accurately determining ascites volume is the crucial first step in clinically treating a patient with malignant ascites. Ultrasonography is commonly used to identify the existence of ascites, and several methods have been proposed to estimate ascites volume. Reportedly, the sum of the depth of ascites at five points (named "five-point method") on three panels of computed tomography images is well correlated to the actual ascites volume and/or abdominal girth. This method is already suited to repetitive assessment due to its convenience compared to the conventional volume rendering method. Meanwhile, a new concept, "Clinical Benefit Response in GC (CBR-GC)", was recently introduced to measure the efficacy of chemotherapy for malignant ascites of GC. CBR-GC is a simple and reliable patient-oriented evaluation system based on changes in performance status and ascites, and is expected to become an important clinical endpoint in future clinical trials. The principal of treatment for GC patients with ascites is palliation and prevention of ascites-related symptoms. The treatment options are various, including a standard treatment based on the available guidelines, cytoreductive surgery with hyperthermic intraperitoneal chemotherapy (HIPEC), laparoscopic HIPEC alone, intravenous chemotherapy, intraperitoneal chemotherapy, and molecular targeting

therapy. Although each treatment option is valid, further research is imperative to establish the optimal choice for each patient.

Key words: Ascites; Clinical benefit; Gastric cancer; Peritoneal dissemination; Paclitaxel

Core tip: Malignant ascites affects approximately 10% of patients with gastric cancer (GC) and poses significant problems for treatment. Accurate and repetitive measurement of ascites volume during treatment is clinically imperative for effective decisions surrounding treatment continuation. Meanwhile, clinical benefit response in GC, a patient-oriented assessment framework of treatment efficacy, should be used in future clinical trials for malignant ascites caused by GC. Although several treatment options have been reported, further studies are mandatory to develop a solid and optimal treatment strategy.

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INTRODUCTION

Malignant ascites caused by gastric cancer (GC) is an accumulation of excess fluid within the abdominal cavity associated with serious clinical problems. First, it is one of the late manifestations of GC, and thus is often accompanied by a severely impaired patient condition[1-3]. A retrospective study of 119 patients with malignant ascites due to GC revealed that 31% of these patients were classed as having as Eastern Cooperative Oncology Group performance status (ECOG-PS) of 3 or more[2]. Generally, such patients have difficulties in receiving standard treatment and show dismal prognosis, with a reported median survival time of 4.6 mo when treated with 5-fluorouracil alone or 5-fluorouracil plus methotrexate[3]. Consequently, these GC patients are often excluded from clinical trials[1] and attract only limited attention with respect to management strategies, thus further veiling the condition and possible treatment of ascites development.

Adequately assessing both ascites volume and treatment efficacy is another clinical problem for patients with malignant ascites due to GC[4]. Because it is difficult to measure the exact nature and extent of disseminated tumors on radiological examination[5], the evaluation of treatment efficacy has empirically relied on changes in the ascites volume. In clinical trials, each protocol had arbitrarily defined the response of ascites to treatment or applied ambiguous definitions of the condition, further complicating inter-trial comparisons. Indeed, the lack of a "standard" method to evaluate treatment efficacy for these patients in daily clinical practice urgently demands the development of a reliable assessment framework.

Despite these documented difficulties, pioneers in this field have successfully conducted several phase Ⅱ studies or retrospectively reported precious results for specific treatment options, and based on these results, patients and clinicians now have expanded treatment options[6]. For instance, long-term survival was achieved after combination therapy of surgical treatment and chemotherapy among selected patients with ascites and GC, although these reports presented limited patient numbers[7-9]. However, the data has not yet been integrated and selecting the most suitable treatment remains a great burden for both patients and clinicians.

In this manuscript, we first review the relevant literature to elucidate the incidence of ascites development among patients with GC. Then, we introduce recently reported methods to measure ascites volume by computed tomography (CT), and explain a new concept for evaluating treatment efficacy based on patient-oriented parameters. Finally, we discuss each treatment option with respect to future directions.

INCIDENCE OF ASCITES DUE TO GC

Data showing the incidence of ascites secondary to GC are scarce and glancing. The development of malignant ascites, an end-stage manifestation of GC, requisitely depends on the tumor stage at diagnosis of primary lesions. Thus, in countries where GC is diagnosed at an earlier stage through validated screening programs[10,11], the incidence of malignant ascites could be relatively low. Contrarily, in countries where less attention is paid to GC due to its lower morbidity, diagnosis of the disease is often delayed until symptoms develop. Therefore, the available literature covers a range of incidence rates of ascites development due to GC across various countries and study periods (Table 1).

Specifically, a retrospective study analyzing more than 7000 patients who underwent gastrectomy in a single Japanese institution from 1960 to 1988 found that 14.2% of the patients developed peritoneal recurrence[10]. Similarly, a Japanese nationwide study in 2009 suggested that 9.9% of 13002 patients who underwent gastrectomy in 2002 died from peritoneal involvement of GC during the 5-year follow-up period[12], while further studies also suggested that approximately 40% of consecutive patients with peritoneal dissemination also showed malignant ascites[13,14]. Thus, these previous data indicated that approximately 4%-5% of all patients undergoing gastrectomy would

Table 1 Incidence of peritoneal dissemination and ascites development due to gastric cancer

Ref.	No. of patients	Period	Country	Status of primary disease	Incidence
Development of peritoneal dissemination					
Nakajima *et al*[10]	7060	1960-1988	Japan	After gastrectomy	14.2%
Nashimoto *et al*[12]	13002	2002	Japan	After gastrectomy	9.9% (related to death)
Development of ascites					
Lello *et al*[15]	356	1980-2004	Norway	At initial diagnosis	6.2%
Yajima *et al*[31]	293	1988-2002	Japan	GC with T2-3 at diagnosis	15.0%
Fang *et al*[16]	5542	2007-2012	China	At initial diagnosis	2.6%[1]
				During the course of disease	3.7%[1]
Kitayama *et al*[14]	83	2006-2008	Japan	Peritoneal recurrence	40.0%
Tahara *et al*[13]	56	1993-1999	Japan	Peritoneal recurrence	46.4%

[1]Diagnosis of malignant ascites is limited to ascites with positive cytology.

be subsequently diagnosed with malignant ascites.

More direct evidence on the incidence of ascites comes from a retrospective analysis in a hospital serving a single, well-defined area of Norway[15]. The authors analyzed 354 patients with clear chart descriptions and identified 6.2% (22/354) as having ascites at the diagnosis of GC. A similar, larger scale retrospective study from China identified ascites in 2.6% of GC patients at the time of initial diagnosis, and in 3.7% of patients thereafter[16]. In these Chinese patients, the diagnosis of malignant ascites was confined to cytology-positive cases, therefore the real prevalence of ascites due to GC would be somewhat higher than that reported[2].

Malignant ascites due to GC is often accompanied by other symptoms related to peritoneal dissemination, a particular recurrence mode of GC[17,18] that can cause obstruction of the gastrointestinal tract, bile duct, and ureter. In addition, half of the patients with peritoneal recurrence have concomitant recurrence sites[17], including lymph nodes, liver, and lung, necessitating the systemic evaluation of each site using imaging modalities. We thus consider that diversity among the accompanying symptoms and conditions of GC patients with malignant ascites could induce factors that hinder clear depiction of these patients in the literature and consequently, could obstruct the establishment of reliable guidelines.

Based on the available data, we estimate that 3%-6% of patients with GC has ascites to some extent at the initial presentation of cancer. Eventually, 10%-15% of those patients treated by curative resection will develop peritoneal recurrence and approximately half of them are likely to develop ascites. Thus, 8%-13.5% of the total number of patients diagnosed with GC will have accompanying malignant ascites. However, the incidence of "massive" malignant ascites that defies conventional treatment remains obscure.

PATHOPHYSIOLOGY

Ascites develops from an imbalance between the production and drainage of peritoneal fluid[19-22]. In adults, the serous membrane covers nearly 2 m^2 of the peritoneal surface[23], and the cavity typically contains 50-100 mL of fluid that turns over at the rate of 5 mL/24 h[24]. Peritoneal fluid is generated by the transudation of plasma from peritoneal capillaries[19], and it serves to lubricate the serous membrane. The fluid eventually drains into the lymphatic system *via* open-ended channels (named stomata) and then into the systemic circulation through the right thoracic duct[24].

Among multiple factors, increased vascular permeability due to vascular endothelial growth factor (VEGF) is considered an important driver of increased ascites production. Zebrowski *et al*[25] demonstrated markedly elevated VEGF levels in malignant ascites including from GC. They further showed augmented permeability of endothelial cells in vitro when cultured with malignant ascites. Another important factor in the development of ascites is matrix metalloproteinase-9 and -2, a key enzyme in tumor cell metastasis to distant organs due to its role in breaking down the tissue matrix. Reportedly, matrix metalloproteinase enhances the release of biologically active VEGF in a time- and dose-dependent manner, and might thus by a key regulator of VEGF in ascites production[26]. In fact, inhibition of matrix metalloproteinase significantly suppressed tumor growth in a rodent model of metastasis[27]. Several other factors could also play a role in the development of malignant ascites during cancer (Table 2), and each could be a potential target of prevention and treatment.

IDENTIFYING AND ASSESSING ASCITES VOLUME

Objectively evaluating the nature and volume of ascites is the first step in treating patients with ascites and peritoneal dissemination of GC. Cytology should be always considered at the initial evaluation because a positive result is diagnostic, while increased levels of carcinoembryonic antigen in ascites suggests the pathological accumulation of peritoneal fluid due to GC[2]. However, it should be noted that the sensitivity

Table 2 Factors influencing development of ascites due to gastric cancer[19-26]

Increased fluid production
Increased vascular permeability due to increased VEGF and/or MMP-2/-9
Neovascularization of peritoneum
Peritoneal inflammation
Increased portal pressure due to tumor metastasis
High protein concentrations in ascites
Lower concentration of serum proteins due to undernutrition
Decreased drainage of peritoneal fluid
Obstruction of lymphatics

MMP: Matrix metalloproteinase; VEGF: Vascular endothelial growth factor.

of these two measures is relatively low[2], and negative results warrant an integrated evaluation of the ascites based on a range of clinical data.

Although the relationship between ascites volume at diagnosis and prognosis remains controversial[14,28], a weak response of ascites to the anti-cancer treatment is well correlated with poor prognosis[1,14], suggesting that frequent and repetitive volume assessment is particularly important for decision making concerning continuation or withdrawal of the ongoing treatment.

ULTRASONOGRAPHY

In this field, the use of endoscopic ultrasonography is increasingly reported[29,30], because of its excellent ability to detect subtle ascites. This modality would therefore be especially beneficial for predicting prognosis in patients with GC, based on patients with ascites apparently having poorer outcomes than those patients without ascites[16,31-33]; however, the technique requires considerable expertise and is invasive for patients. Abdominal ultrasonography is often used in the emergency room and daily clinical practice due to its convenience, and thus is likely to first detect ascites. Recently, ultrasonography technologies allowed development of much smaller devices, which will eventually eliminate unnecessary further confirmatory examination with invasive modalities[34].

Indeed, as early as 1996 Inadomi *et al*[35] developed a protocol to measure ascites volume with ultrasonography, in patients with portal hypertension. They regarded the ascites in the abdominal cavity as a fluid retained in the base of a large sphere, and developed an algorithm accordingly using two variables: the ascites depth, defined as the distance to the deepest point of the ascites, and abdominal circumference. The calculated value proved to be well correlated with the ascites volume determined by distribution (dilution) of radiolabeled tracer. However, this method had the remaining problem that patients needed to remain in a prone position on their hands and knees for 10 min, and it is not clear whether it could be used in patients with postoperative intraperitoneal adhesions.

More recently, Irshad *et al*[36] reported notable findings of correlation between the smallest depth of ascites on ultrasonography and the subsequent drained volume of ascites. They conducted 60 paracenteses in 29 patients after evaluating the length between the most superficial bowel loop and the abdominal wall. They found that the length measured by ultrasonography was well correlated to the amount of drained fluid, and concluded that ultrasonography could successfully estimate the ascites volume. Establishment of a validated volume-measuring method based on ultrasonography would obviously be a great benefit for patients who require frequent monitoring, and thus further development of these initial studies are eagerly awaited.

FIVE-POINT METHOD ON CT

The recent development of multi-detector CT permits small amounts of ascites to be detected[31,32] and thus imaged by reconstruction of three-dimensional imaging. Although a volume-rendering algorithm applied to such imaging would enable accurate assessment of ascites volume, the procedure requires an appreciable amount of time regardless of the operator expertise. To reduce the burden, Oriuchi *et al*[4] developed a very simple method to estimate ascites volume using the horizontal plane of CT imaging. In this technique, ascites depth was measured at five points on three CT images and the sum of measurements for each patient was multiplied by 200 (Figure 1). The estimated ascites volume was then correlated with the volume calculated with 3D-CT in patients having > 300 mL of ascites. The authors reported that this protocol was reliable even in patients with a history of surgical intervention that might cause changes of their ascites distribution due to adhesion. The accuracy of this method was confirmed by two following studies: one for assessing the ascites due to GC[1] and one for assessing ascites due to a perforated peptic ulcer of the upper gastrointestinal tract[37].

Of note, the horizontal plane of CT imaging at the level of the superior mesenteric artery occasionally does not depict the spleen, and ascites can occur in patients who undergo splenectomy with gastrectomy. In such cases, volume assessment could instead use the distance from the inner surface of the abdominal wall to the surface of an internal organ at a defined line (Figure 1). This altenative method is also reliable for assessing chronological changes in ascites volume (personal communication with Dr. Oriuchi). Despite the apparent limitation of only a small number of patients examined thus far and the lack of explicit evidence for using an alternative measuring parameter rather than the standard one of the distance between spleen and abdominal wall, this CT-based method is fascinating and warrants further exploration.

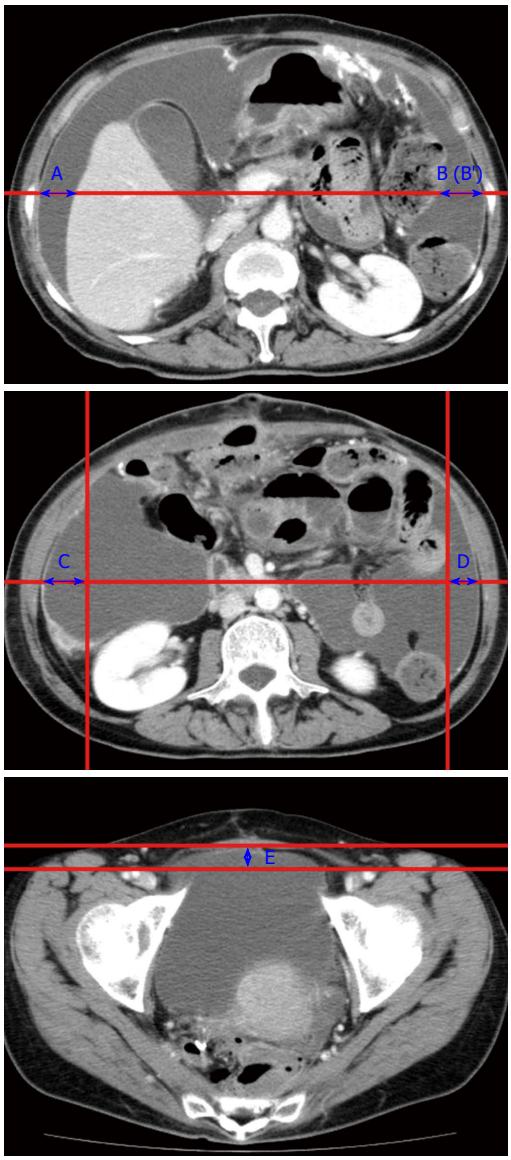

Figure 1 Five-point method to measure ascites volume. Upper: Line between the bilateral antero-posterior mid-points of the abdominal wall is drawn at the plane of the root of the superior mesenteric artery. The distances between the inner surface of the right abdominal wall and the liver (A cm), and between the inner surface of the left abdominal wall and spleen (B cm) are obtained. When spleen is not observed on this plane, the distance between the left abdominal wall and margin to the ascites and internal organs are measured (B' cm); Middle: The lower pole of the left kidney is observed on this plane. The sagittal line from the bilateral paracolic gutter, and between the bilateral antero-posterior midpoints of the abdominal wall is drawn. The distances C (cm) and D (cm) are thus obtained. Lower: A line between the anterior sides of the bilateral femoral artery is drawn. The distance between the inner surface of the abdomen (at the middle) and the line is obtained (E cm). The ascites volume is calculated by the equation of (A + B + C + D + E) × 200 (mL).

CLINICAL ENDPOINT OF TREATMENT FOR ASCITES

Because the peritoneal dissemination sparsely distributes in the abdominal cavity rather than forming a large mass, patients with malignant ascites often lacks measurable lesions. In addition to the ascites volume, the assessment of treatment efficacy in such cases has to be based on the integrated clinical information[38]. Standardized evaluation methods are required to select the patients who should continue the current treatment, and identify the patients who should receive other treatments because of disease progression. The candidate for the clinical endpoint of the treatment could be an improvement of quality of life (QOL) measured by established questionnaires. However, the assessment is often difficult to achieve due to its complexity; Badgwell *et al*[39] courageously demonstrated the difficulty of conducting self-reporting assessment of QOL using Functional Assessment of Cancer Therapy-General (FACT-G)[40]. Only 39% of the patients with incurable cancer and gastrointestinal obstruction completed the questions concerning quality of life and treatment satisfaction at one-month follow up. Based on our similar clinical experiences and observations, we noticed the need for new convenient method to evaluate the treatment efficacy for malignant ascites due to GC, and "Clinical Benefit Response in GC (CBR-GC)" was proposed[1,41].

CONCEPT OF CLINICAL BENEFIT RESPONSE IN GC

CBR is an established palliative endpoint for gastrointestinal tract malignancy that is particularly applied in clinical trials for the treatment of pancreatic cancer[42-44]. CBR is based on three clinical factors: the change in pain, Karnofsky PS, and body weight, although because of the hierarchical structure of CBR, its use is slightly complicated and vulnerable to missing data[44]. In contrast, CBR-GC is a newly proposed concept for evaluating the response of malignant ascites due to GC to anti-cancer therapy. This evaluation system has two major components: (1) change in ECOG-PS; and (2) change in ascites evaluated using the patient-oriented method.

Change in ascites (response) with treatment (lateral axis of Figure 2) is determined by abdominal girth and intensity of palliation, which comprises the use of diuretics and paracentesis. When the abdominal girth decreases in volume and the intensity of palliation is not increased, the response of ascites is termed "Positive". Meanwhile, either or both an increase in abdominal girth and increased intensity of treatment is classed as a "Negative" response to treatment. Comprehensive judgment is then based on the general assessment of ascites response and ECOG-PS. Positive CRB-GC is defined by an improvement of either or both ascites and ECOG-PS without deterioration of any parameter. If the CRB-GC is not positive, the status is determined as negative.

CLINICAL USE OF CBR-GC

When CRB-GC was used to evaluate the efficacy of paclitaxel treatment in patients with malignant ascites

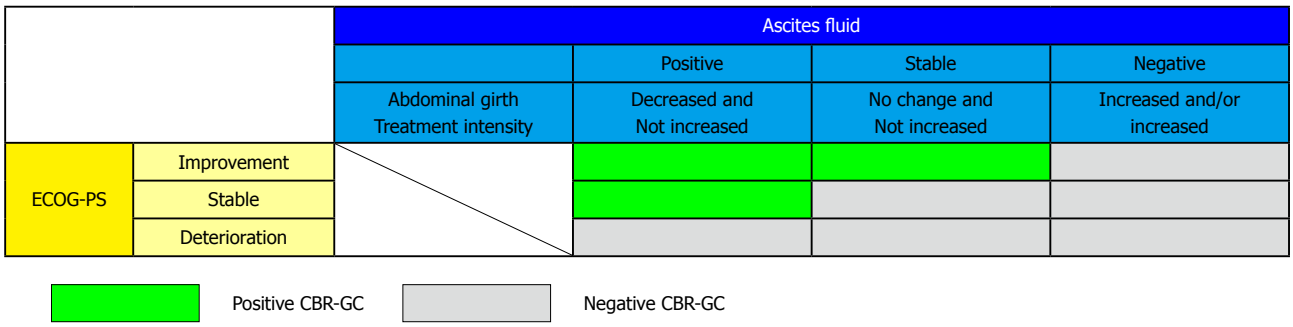

Figure 2 Clinical benefit response in gastric cancer. CBR-GC is defined by the ascites response to treatment (horizontal axis) and ECOG-PS (vertical axis). Response of ascites is judged by a combination of abdominal girth and treatment intensity. CBR-GC: Clinical benefit response in gastric cancer; ECOG-PS: Eastern Cooperative Oncology Group performance status.

due to GC, 39.1% had a positive CRB-GC, suggesting that CRB-GC could serve as a prognostic predictor in such patients. The median survival time of patients with positive CRB-GC was 9.9 mo, while that of patients with negative CRB-GC was 3.6 mo (*P* value not shown).

The possible limitation of CRB-GC is that subtle alleviation of ascites-related symptoms, which could be of real importance for patients, might be overlooked when solely based on these two factors of ascites response and ECOG-PS, when clinically, symptom improvement should signal continuation of the treatment to prevent deterioration in the patient's condition. However, CRB-GC could become negative when both ascites and ECOG-PS are "stable". Thus, further study is necessary to clarify whether this new evaluation system is adequately correlated with subtle symptom alleviation. As suggested by the authors, the relationship between improvement in patient quality of life and CRB-GC status should be addressed. Additionally, the abdominal girth seems precarious compared to the volume measurement by CT, and the threshold of decrease or increase in abdominal girth has to be clearly defined. Although, the authors found that the 5% of possible error did not change the primary results, the exact threshold of change in abdominal girth that determines "no change" should be verified.

TREATMENT

Choosing a treatment option for GC with malignant ascites also relies on the patient's general condition. Despite the presence of ascites, standard treatment as per the guidelines[45-47] should be the first choice when the patients are motivated enough and in a good general condition without insufficiency of vital organs. While many alternative treatment options have been reported, evidence-based guidelines for each remain to be developed.

SYSTEMIC CHEMOTHERAPY

Paclitaxel monotherapy

Pharmacokinetic studies of paclitaxel shows that concentration of this drug in ascites remains within the optimal range up to 72 h after intravenous administration at the dose used in a weekly regimen[48], while a similar dose of paclitaxel maintains the serum concentration above the minimum effective concentration at least for 24 h[49]. The bulky molecular structure, molecular weight, and high affinity to proteins in ascites probably delays the clearance of paclitaxel from the peritoneal cavity[48], possibly explaining its efficacy on ascites and peritoneal dissemination. Imamoto *et al*[1] conducted a phase Ⅱ study focusing on the efficacy of paclitaxel monotherapy in patients with malignant ascites due to GC, recruiting 64 patients with a median ascites volume of 2796 mL (range, 122 mL to 7623 mL). This paclitaxel monotherapy regimen achieved volume reduction in 31.1% of patients (Table 3) and 39.1% of the patients experienced positive CBR-GC. We consider that the low frequency of adverse events and the treatment efficacy warrants applying this treatment for a wider range of patients[1,6,50-52].

Docetaxel

Pharmacokinetics study of docetaxel after synchronous administration with fluoropyrimidines demonstrated that docetaxel concentration in ascites remained high up to 24 h after the intravenous administration[53]. This synchronous administration of two different anti-cancer agents achieved a median survival time of 7.2 mo in 24 patients with GC and malignant ascites. However, the author noted that the concentration of docetaxel in ascites was not correlated with the reduction in ascites, leaving the possibility of a significant influence due to the fluoropyrimidine.

Evidence concerning the direct effect of docetaxel monotherapy on malignant ascites due to GC is not available, to our knowledge; however, the weekly docetaxel monotherapy achieved disease stabilization (defined as a complete response, partial response, or stable disease lasting more than 100 d) in 36% of elderly patients or patients with impaired performance status, with an acceptable safety profile[54]. In addition, several case reports demonstrated its efficacy in treating patients with peritoneal recurrence and/or malignant ascites refractory to paclitaxel[55,56]. Gligorov

Table 3 Systemic chemotherapy for patients with malignant ascites due to gastric cancer

Ref.	Regimen	No. patients	ECOG-PS 0/1/2	Ascites volume	Prior chemotherapy	Main findings	Grade 3 or more AE
Imamoto *et al*[1]	Paclitaxel[1]	64	24/28/12	Mean: 2906 mL (range: 122-7623 mL)	37	Positive CRB-GC: 39.1% MST 5.2 mo Positive CRB-GC: 9.9 mo Non-response: 3.6 mo	Neutropenia: 19.1% Hyponatremia: 19.1% Anorexia: 22.2%
Hironaka *et al*[51]	Paclitaxel[1]	38 (21)	12/15/11	ND	38	Ascites volume reduction: 5/21	Neutropenia: 32% Leukopenia: 29% Death within 30 d of the last administration
Takeyoshi *et al*[58]	Paclitaxel Doxifluridine[2]	24	8/4/2012	ND	14	MST: 7.2 mo 1-yr OS: 29.2 and RR: 41.7%	Leukopenia: 25% Elevated alt: 12.5%
Iwasa *et al*[59]	Paclitaxel	25	1/19/5	Non: 1	7	Ascites volume reduction: 44%	Neutropenia: 12%
	Fluorouracil Leucovorin[3]			Mild: 6 Moderate: 2 Masive: 16		MST: 8.0 mo	Anemia: 12% Hyponatremia: 16% Anorexia: 16%
Oh *et al*[60]	mFOLFOX-4[4]	48	0-1/2: 26/22	ND	27	MST: 8.4 mo Ascites volume reduction: 35.4%	Neutropenia 18.8% (per cycle) Nausea: 6.3% Febrile neutropenia: 2.6% (per cycle)
Yamao *et al*[38]	Methotrexate Fluorouracil[5]	37	8/24/5	ND	0	Ascites volume reduction: 35.1%	Neutropenia: 27% Elevated total bilirubin: 24.3% Anemia: 24.3%
Nakayama *et al*[62]	Methotrexate	47 (23)	10/13/24	ND	8/47	Ascites volume reduction: 15/23	Leukopenia: 21.3%
	Fluorouracil Cisplatin[6]					MST 211 d	Neutropenia: 19.1% Nausea: 2.1% Anorexia: 2.1%

[1]Eighty mg/m^2 of paclitaxel intravenously on day 1, 8, 15, every 4 wk; [2]80 mg/m^2 of paclitaxel intravenously on day 1, 8, 15, every 4 wk and doxifluridine 533 mg/m^2 at day 1-5, every week; [3]500 mg/m^2 of fluorouracil, 250 mg/m^2 of leucovorin, and 60 mg/m^2 of paclitaxel intravenously on day 1, 8, 15, every 4 wk; [4]85 mg/m^2 of oxaliplatin on day 1, 20 mg/m of leucovorin, 400 mg/m^2 of fluorouracil on day 1 and 2, followed by 600 mg/m^2 of fluorouracil over 22 h, every 2 wk; [5]100 mg/m^2 of methotrexate and 600 mg/m^2 of fluorouracil intravenously, every week; [6]30 mg/m^2 of methotrexate, 600 mg/m^2 of fluorouracil on day 1 and 8, 6 mg/m^2 cisplatin on days 1-14, every 4 wk. AE: Adverse event; ND: Not described; ECOG-PS: Eastern Cooperative Oncology Group performance status.

et al[57] cautiously summarized the difference between taxanes, and found favorable molecular features, pharmacokinetics, and drug interactions of docetaxel as an anti-cancer drug. Thus, a phase Ⅱ study is urgently needed to adequately determine the efficacy and safety of docetaxel monotherapy, ideally in comparison with paclitaxel, for patients with malignant ascites due to GC.

Combination therapy

The advantage of combination therapy over monotherapy has to be more definitively proved in clinical trials before wide take-up by clinicians, with several one-arm phase Ⅱ studies favouring monotherapy[58-60]. Takeyoshi *et al*[58] performed a Phase Ⅱ trial to evaluate combination therapy of paclitaxel and doxifluridine, an intermediate metabolite of capecitabine, and found that the treatment yielded an ascites response rate of 41.7% and MST of 215 d (equivalent to 7.2 mo) in 24 patients, with a 25% occurrence rate of grade 3/4 leukopenia. The accompanying pharmacokinetics study revealed that the ascites concentration of paclitaxel following such therapy was within the therapeutic range up to 72 h, which is consistent with previous reports of monotherapy[48]. Similarly, paclitaxel and 5-fluorouracil achieved a median overall survival of 8.0 mo and ascites response rate of 44% in patients with massive ascites or inadequate oral intake[59].

Korean scholars reported the effectiveness of modified FOLFOX-4 in patients with malignant ascites due to GC with the protocol regime applied as first-line, second-, or third-line treatment[60]. A decrease or disappearance of ascites was observed in 35.4% (17/48) patients, with a median overall survival of 8.4 mo; however, the treatment could be harsh because grade 3 neutropenia was not uncommon (0.188 event per cycle) and grade 4 febrile neutropenia occurred 6 times among 233 treatment cycles.

In terms of methotrexate, two phase Ⅱ clinical trials explored the efficacy of combination therapy of methotrexate and 5-fluorouracil[38,61], and found complete disappearance of ascites or apparent reduction of ascites in 35%-54% of the patients. A study of more complicated combination chemotherapy with methotrexate, a-fluorouracil, and low-dose cisplatin for diffuse-type advanced and recurrent GC (KDOG9501) recruited 47 patients, 23 patients of which had significant amounts of ascites[62]. The results showed that 4 out of 23 ascites disappeared, while 11 patients had decreased ascites, thus 65.2%

of the patients with ascites experienced improvement. The median survival time was reported to be 211 d (equivalent to 7.0 mo). However, a recent phase Ⅲ clinical trial including 237 patients with peritoneal dissemination (among them, 171 patients had malignant ascites) suggested that the combination therapy is not superior to 5-fluorouracil monotherapy in terms of overall survival[63].

INTRAPERITONEAL CHEMOTHERAPY

Anti-cancer drugs administered into the peritoneal cavity penetrate the tumor nodules by passive diffusion. Because penetration depth is limited[64], such intraperitoneal administration is not sufficient to treat larger nodules, and combinations of intravenous and intraperitoneal administration of anti-tumor drugs have been attempted to treat such cases. Cisplatin is an effective agent to treat GC when it is administered intravenously. However, intraperitoneal administration of cisplatin is not a common practice due to its proven lack of benefit as adjuvant chemotherapy[65], probably due to immediate clearance from the peritoneal cavity. To prolong the effect of cisplatin within the abdominal cavity, a new drug delivery system has to be developed. In this context, the commonly applied agents are taxanes. For instance, a phase Ⅱ randomized trial comparing intravenous and intraperitoneal paclitaxel administration has been implemented by Kodera *et al*[66] (UMIN000002957) under the supervision of Ministry of Health, Labor and Welfare as an advanced medical treatment project of the government.

For treatment of patients with malignant ascites due to GC, Kitayama *et al*[14] evaluated the efficacy of synchronous administration of intravenous and intraperitoneal paclitaxel, and oral administration of S-1. The study enrolled 33 patients with ascites due to GC, 9 of which had more than 2500 mL of ascites before treatment. After the initiation of the treatment, 70% of the cases showed > 50% reduction in ascites volume and an associated improvement in prognosis, with a median survival time of 455 d. Another study from the same group further showed that the combination treatment could be safely followed by curative resection (gastrectomy) in selected patients[7]. In such cases, the one-year survival rate was 82% with a median survival time of 26.4 mo. Meanwhile, the patients with refractory ascites against treatment had a median survival time of 12.1 mo. As discussed by the authors, the benefit of salvage gastrectomy remains unclear; however, such treatment is apparently beneficial for selected patient because 5-year survival could be achieved by this sequential treatment. The frequent adverse events for this regimen were neutropenia and leukopenia (25% of patients), while occlusion or infection of the access port is also conceivable with the intraperitoneal chemotherapy.

HYPERTHERMIC INTRAPERITONEAL CHEMOTHERAPY AND CYTOREDUCTIVE SURGERY

HIPEC after cytoreductive surgery has received increasing attention due to its efficacy in treating peritoneal dissemination of GC[67,68]. We speculate that many of the patients in these cited studies had some degree of malignant ascites; however, there was little focus on the efficacy of HIPEC with/without cytoreduction in patients with significant amounts of malignant ascites. Yang *et al*[8] explored the clinical benefit of this combination therapy in 28 patients with GC and heavy ascites or peritoneal carcinomatosis. Of these patients, 20 had ascites and positive cytology, and the detailed information of 12 patients with ascites was available. These patients died at 2, 3, 8, 8, 9.5, 9.5, 10.5, and 29.5 mo after surgery, and survival was confirmed at 3, 5, 9, and 19 mo, yielding a median survival time of 9.5 mo (95%CI: 7.6-11.4 mo). Although the beneficial effect of this treatment seems marginal for the majority of patients, long-term survival was observed and this is generally difficult to achieve with other modalities. Unfortunately, the study did not elucidate the preoperative factors significant for long-term survival, and thus patient selection would become an important issue to be solved. In addition, the related complications and even mortality associated with this combination regime might attenuate its attractiveness.

Another of HIPEC demonstrated that only 14 out of 45 patients with peritoneal dissemination of GC could undergo optimal cytoreductive surgery, (meaning no visible tumor residue or only residual nodules < 2.5 mm). The authors performed HIPEC only in these 14 patients because the penetration of chemotherapeutic agents into tissue nodules is limited and they considered that performing HIPEC in patients with large residual nodules would not be beneficial[9]. Consequently, the optimal treatment group with cytoreduction and HIPEC showed longer median survival than cytoreduction only (median survival time of 18 mo *vs* 6 mo, $P = 0.0007$). They also found that the preoperative risk factors of incomplete cytoreduction were retention of ascites and preoperative malnutrition (prognostic nutrition index). Indeed, attempting to perform cytoreductive surgery with HIPEC on patients with GC and ascites is in and of itself a challenging task.

Because cytoreductive surgery and HIPEC could be too damaging for patients with GC and an already poor general condition, reduced invasiveness was attempted by using laparoscopic intraperitoneal hyperthermic chemotherapy. Reports of this modification are still scant and the survival benefit remains unclear[69-71]; however, the recurrence of ascites development was suppressed in the majority of studied cases and thus exploration of this treatment might be justified.

MOLECULAR TARGETING THERAPY

Molecular targeting therapies such as bevacizumab[72], cetuximab[73,74], panitumumab[75], and everolimus[76] have not shown significant survival benefit in patients with GC. Thus, these agents are unlikely to be used vigorously for malignant ascites secondary to GC. Trastuzumab is a humanized monoclonal antibody targeting human epidermal growth factor receptor type 2 (HER-2), and some clinical trials demonstrated the efficacy and safety of combination therapy with trastuzumab, cisplatin, and fluoropyrimidines[77,78]. Although the protocols used did not exclude the existence of ascites, data concerning ascites response was seldom described. An ongoing phase Ⅱ trial focusing on patients with unmeasurable lesions of HER2-positve GC (UMIN000007941) may give some further indication for this treatment of malignant ascites.

Recently the clinical benefit of catumaxomab was reported. This chimeric antibody of mouse-derived anti-EpCAM Fab region and a rat antiCD3 Fab can recognize epithelial cell adhesion molecule-expressing tumor cells, CD-positive T cells, and Fcγ receptor-positive immune cells, and improved the quality of life in patients with ascites due to several kinds of malignancies compared to best supportive care[79,80]. Because this treatment did not prolong survival time, catumaxomab should be considered only in patients whose cancer is difficult to treat by conventional regimes. Meanwhile, the efficacy of combination therapy with catumaxomab and chemotherapy, which has minimum toxicity, such as monotherapy of taxanes, should be explored for severely deteriorated patients with GC and malignant ascites, as suggested by Imamoto *et al*[1].

CONCLUSION

Malignant ascites is a common manifestation of end-stage GC, affecting approximately 10% of patients. Because prognosis in these patients can be predicted during treatment by changes in the ascites volume, repetitive and objective evaluation of such volumes is critically important to maximize patient outcomes. Meanwhile, CBR-GC can assess the treatment efficacy based on changes in ECOG-PS and ascites due to anti-cancer treatments, and should be used for such assessments in future clinical trials.

For patients with malignant ascites due to GC, a guidelines-based standard treatment should be the first considered. In many cases, however, an alternative treatment has to be chosen from the wide range of options, based on the patient's general condition, their understanding of the disease, and support from personal circumstances. Despite the progress in this field, the best supportive care is often the only thing that can be offered to patients suffering from end-stage GC. Thus, more understanding of this condition and development of evidence-based treatment strategies, together with new treatment options, are necessary.

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TOPIC HIGHLIGHT

2015 Advances in Gastric Cancer

Companion diagnostics for the targeted therapy of gastric cancer

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Abstract

Gastric cancer is the fourth most common type of cancer and represents a major cause of cancer-related deaths worldwide. With recent biomedical advances in our understanding of the molecular characteristics of gastric cancer, many genetic alterations have been identified as potential targets for its treatment. Multiple novel agents are currently under development as the demand for active agents that improve the survival of gastric cancer patients constantly increases. Based on lessons from previous trials of targeted agents, it is now widely accepted that the establishment of an optimal diagnostic test to select molecularly defined patients is of equal importance to the development of active agents against targetable genetic alterations. Herein, we highlight the current status and future perspectives of companion diagnostics in the treatment of gastric cancer.

Key words: Companion diagnostics; Gastric cancer; Human epidermal growth factor receptor 2; Fibroblast growth factor receptor; Hepatocyte growth factor receptor

Core tip: Companion diagnostics are *in vitro* clinical laboratory assays designed to predict the efficacy of treatment using biomarker-based assessments. For patients with gastric cancer, immunohistochemistry for human epidermal growth factor receptor 2 (HER2) overexpression and fluorescence *in situ* hybridization for *HER2* amplification are the only approved companion diagnostic devices. In this era of targeted therapy, the concurrent development of companion diagnostic techniques is critical for the success of novel therapeutics. Furthermore, the successful co-development of drug and companion diagnostics requires a thorough molecular understanding of both tumor biology and the mechanisms of drug actions.

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INTRODUCTION

Gastric cancer (GC) is the fourth most common type of cancer and represents a major cause of cancer-related deaths worldwide[1,2]. Surgery is the curative treatment option for patients with localized GC and the survival of patients with resectable GC has improved to 5-year survival rates of 72%-78% in East Asia with enhanced efficacy imparted by adjuvant treatment[3,4]. However, with the exception of several countries in East Asia where national screening programs for the detection of GC are conducted, such as South Korea and Japan, most patients initially present with inoperable or metastatic disease[2,5]. Although no single standard cytotoxic chemotherapy regimen has been established, doublet or triplet regimens that include fluoropyrimidine and platinum have been accepted as the current standard treatments for patients with inoperable or metastatic GC[6-9]. The activities of taxanes and irinotecan have been demonstrated and these compounds are widely used as second-line chemotherapy[10-12]. However, the prognosis of patients with metastasis or inoperable GC remains poor and is associated with an overall survival of approximately 1 year[6-9].

Recent advances in biomedical research have advanced our understanding of the molecular characteristics of GC, leading to the identification of many genetic alterations as potential targets for its treatment[13,14]. Trastuzumab, a monoclonal antibody against HER2, and ramucirumab, a fully human IgG1 monoclonal antibody, VEGFR-2 antagonist, have demonstrated survival benefits in randomized phase 3 trials and been approved for the treatment of GC[15-17]. Multiple novel agents are now under development as the demand for active agents that can improve the survival of GC patients is constantly increasing. Based on lessons from previous trials of targeted agents, it is now widely accepted that the establishment of an optimal diagnostic test to select molecularly defined patients is as important as the development of active agents against targetable genetic alterations. In this review, we highlight the current status and future perspective of companion diagnostics in GC.

CURRENT STATUS OF COMPANION DIAGNOSTICS IN GC

The history of companion diagnostics began with the United States Food and Drug Administration (FDA) approval of an immunohistochemistry (IHC) assay (HercepTest™, Dako Denmark A/S, Glostrup, Denmark) for HER2 protein overexpression in 1998[18]. Companion diagnostics are generally known as *in vitro* clinical laboratory assays designed to predict the efficacy of treatment through the assessment of biomarkers[19]. In a draft guidance issued by the United States FDA in 2011[20], companion diagnostics were defined as essential devices for the (1) identification of patients who are most likely to benefit from a particular therapeutic product; (2) identification of patients likely to be at increased risk of serious adverse reactions as a consequence of treatment with a particular therapeutic product; and (3) monitoring of responses to treatment so that treatments can be adjusted to achieve improved safety or efficacy. Previously, this has been given various names, such as pharmacodiagnostics, theranostics, and pharmacogenomic biomarkers, but the term "companion diagnostics" is now more commonly used and has been adapted by the United States FDA and the European Union (EU)[19]. Companion diagnostics have a central role in drug development as techniques with analytical validity allow investigators to conduct clinical trials using an enriched study design, which is likely both to reduce sample sizes and costs, and to increase success rates[21]. Additionally, a key goal of clinical precision medicine is to prescribe the right drug for the right patient. The United States FDA has approved IHC assays, *in situ* hybridization, and target DNA mutation analyses as companion diagnostics for cancer[18]. For patients with GC, the HercepTest™ for IHC assessment of HER2 overexpression and the HER2 fluorescence *in situ* hybridization (FISH) PharmDx™ Kit (Dako Denmark A/S) for the detection of gene amplification are the only approved companion diagnostic devices that are based on a successful randomized phase 3 ToGA trial[15,18].

HER2 pathway signaling

HER2 overexpression or amplification has been reported in approximately 20% of GC cases[22-25]. In contrast to breast cancer, in which HER2-positivity is significantly correlated with a poor prognosis, the prognostic significance of HER2 overexpression or its amplification in GC has been the subject of controversy[23,26]. This issue might be attributable to the unique characteristics of HER2 expression patterns in GC, such as discrepancies in the frequency of HER2 overexpression (30% in intestinal type *vs* 6% in diffuse type) according to the Lauren's classification, a well-known prognostic factor in GC, and variability in IHC staining that can indicate tumor heterogeneity in HER2 expression, particularly in IHC 2+ cases[25].

Based on the efficacy of trastuzumab in preclinical models of GC[27] and its marked success in HER2-positive breast cancer, a randomized phase 3 ToGA trial that compared chemotherapy with or without trastuzumab was conducted[15]. When the ToGA trial was planned, validated test methods and scoring systems for HER2 status were widely available for breast cancer, but not GC. Therefore, a validation

Table 1 Human epidermal growth factor receptor 2 scoring criteria for gastric cancer[15,23]

Score	Surgical specimen-staining pattern	Biopsy specimen-staining pattern	HER2 overexpression assessment
0	No reactivity or membranous reactivity in < 10% of tumor cells	No reactivity or no membranous reactivity in any tumor cell	Negative
1+	Faint/barely perceptible membranous reactivity in ⩾ 10% of tumor cells; cells are reactive only in part of their membrane	Tumor cell cluster with a faint/barely perceptible membranous reactivity irrespective of percentage of tumor cells stained	Negative
2+	Weak to moderate complete, basolateral, or lateral membranous reactivity in ⩾ 10% of tumor cells	Tumor cell cluster with a weak to moderate complete, basolateral, or lateral membranous reactivity irrespective of percentage of tumor cells stained	Equivocal
3+	Strong complete, basolateral, or lateral membranous reactivity in ⩾ 10% of tumor cells	Tumor cell cluster with a strong complete, basolateral, or lateral membranous reactivity irrespective of percentage of tumor cells stained	Positive

HER2: Human epidermal growth factor receptor 2.

study was performed to establish a HER2 scoring system for GC to identify suitable patients for enrollment in the trial[23]. In that study, which used the HercepTest™ and FISH PharmDx™ Kit, it was noted that tumor heterogeneity and basolateral membrane staining were more common in GC than in breast cancer. That study concluded that the scoring system of HER2 IHC assessment using the HercepTest™ in breast cancer should be applied to GC with some modifications because of incomplete reactivity in tumor cell membranes and tumor heterogeneity, which are both frequently observed in GC (Table 1). It was also recommended that both IHC and FISH testing should be used to select patients for the ToGA trial because of differences in results between GC and breast cancer. Based on this validation study of the HER2 scoring system in GC, patients were enrolled in the trial if their tumor samples were scored as 3+ by IHC or as FISH-positive (a HER2: CEP17 [centromeric probe 17] ratio ⩾ 2)[15].

In the ToGA trial that enrolled 594 chemotherapy-naive patients with GC, the addition of trastuzumab significantly improved the efficacy of chemotherapy with 2.7 mo of benefit in median overall survival (13.8 mo for chemotherapy with trastuzumab *vs* 11.1 mo for chemotherapy alone)[15]. After the success of the ToGA trial, trastuzumab became the first biological agent approved for the treatment of GC, and the combination of trastuzumab and cytotoxic chemotherapy is now considered as a standard treatment for metastatic or recurrent HER2-positive GC. Although the results of the FISH and IHC assays have been shown to be highly correlated[24], HER2 overexpression assessed by IHC was more significantly correlated with the efficacy of trastuzumab, irrespective of the FISH results. Indeed, the median overall survival was 10.0 mo in the IHC 0 or 1+/FISH-positive subgroup, whereas it was 16.0 mo in the IHC 2+ or 3+/FISH-positive subgroup[15]. This improved efficacy of trastuzumab in HER2 IHC 2+/FISH-positive or IHC 3+ GC patients was supported by the findings of subsequent phase 2 trials that used trastuzumab-containing regimens against GC[28,29]. Ongoing trials for novel HER2-targeted therapies, such as pertuzumab[30] and T-DM1 (NCT01641939), now use this selection criterion (*i.e.*, IHC 2+/FISH-positive or IHC 3+) for the inclusion of patients. Additionally, IHC staining is recommended as the initial testing modality for all GC patients to define HER2 positivity in daily clinical practice[24,31].

Lapatinib is a tyrosine kinase inhibitor that blocks both epidermal growth factor receptor (ErbB1) and HER2, which has been approved for the treatment of HER2-positive breast cancer after progression to trastuzumab[32]. The efficacy of lapatinib in GC was investigated in two large randomized phase 3 trials[33,34]. The TyTAN trial compared the combination of lapatinib plus paclitaxel with paclitaxel alone in a second-line setting for 281 patients with *HER2*-amplified GC that was assessed by FISH (HER2: CEP17 ratio ⩾ 2)[33]. That trial failed to show significant improvements in overall survival with the addition of lapatinib in a second-line setting. Despite the negative results in an intent-to-treat population, subgroup analyses revealed that lapatinib was significantly associated with better overall survival in patients with HER2 IHC 3+ GC. This finding suggested that the efficacy of lapatinib might correlate with HER2 overexpression as assessed by IHC, which was also shown in a ToGA trial for trastuzumab. As this trial only used FISH for patient selection, more patients with HER2 IHC 0/1+ (35%) were included than in the ToGA trial in which 22% of patients were HER2 IHC 0/1+[15,33]. Therefore, improper selection of the target patient population might represent a potential reason for negative results, although the lack of efficacy of lapatinib against HER2-positive GC should also be considered. The TRIO-013/LOGiC trial tested first-line capecitabine plus oxaliplatin with or without lapatinib in patients with HER2-amplified or overexpressed GC, which was defined as IHC2+ and FISH amplified, or IHC 3+, or FISH, CISH, or SISH amplified[34]. In this trial of 487 patients, the addition of lapatinib did not improve overall survival, consistent with the results of the TyTAN trial. Moreover, subgroup analyses of this study did not show a correlation between IHC and outcomes with lapatinib, which contradicted the

TyTAN trial findings. The lack of efficacy with lapatinib in these trials might be a consequence of negative interactions between lapatinib and partner cytotoxic chemotherapy agents, insufficient activity of lapatinib on HER2-positive GC, or improper selection of the patient population.

In addition to the IHC HercepTest™ and FISH PharmDx™ used in the ToGA trial, alternative assays or techniques to assess HER2 status have been evaluated. In the subset analysis of the TRIO/LOGiC trial, the results of the PathVysion HER2 FISH probe (Abbott Molecular Inc., IL) were highly correlated with those of HER2 FISH PharmDx™ in the central laboratory, with rates of positive agreement of 97.9% and negative agreement of 99.1%[35]. In a retrospective analysis, there was a high concordance of IHC staining results between the HercepTest™ (polyclonal antibody) and Pathway (monoclonal antibody; Ventana Medical System, Tucson, AZ) for GC patients[36]. Among various methods used to assess *HER2* gene amplification, silver *in situ* hybridization (SISH), a bright field *in situ* hybridization (ISH) method, has been suggested to be a valid alternate option for GC, as previous studies showed 94-100% concordance with FISH findings[36-38].

Hepatocyte growth factor receptor pathway signaling

Although there are no approved companion diagnostics for detecting activation of the hepatocyte growth factor receptor (MET) signaling pathway, it represents one of the most widely investigated biomarkers in GC[39-41]. The role of the MET signaling pathway in tumorigenesis and metastasis has been well documented[42] and MET overexpression or amplification has been suggested to be a negative prognostic marker in GC patients[40]. The preclinical activity of MET inhibitors against MET-amplified or overexpressed GC has also been well established[39]. Multiple drugs that target MET signaling pathways are now in the early and late phases of clinical trials for GC patients. Along with the early clinical development of these agents, efforts to define biomarkers predictive of the efficacy of these agents have been ongoing. Onartuzumab (MetMab) is an anti-c-MET monoclonal antibody and, as MET overexpression has been associated with increased efficacy of onartuzumab in a randomized phase 2 trial for patients with lung cancer[43], MET overexpression assessed by IHC was selected as a marker to enrich patients with MET-positive tumors in trials for GC. A randomized phase 2 trial was conducted for patients with HER2-negative GC to compare modified FOLFOX6 plus onartuzumab with modified FOLFOX6 alone[44]. In that trial, the addition of onartuzumab did not improve progression-free survival in either an unselected population or in MET-positive patients defined by IHC (⩾ 50% of a tumor with moderate to strong intensity staining on central review). Moreover, there was no correlation between the efficacy of onartuzumab and the intensity of MET expression or different definitions for MET positivity (⩾ 90%). Rilotumumab, a hepatocyte growth factor (HGF)-targeted monoclonal antibody, was investigated in combination with epirubicin, cisplatin, and capecitabine (ECX) in a randomized phase 2 trial for GC[45]. In that trial, two different doses of rilotumumab (7.5 and 15 mg/kg) were tested and the addition of rilotumumab was significantly associated with improved progression-free survival [5.7 mo in both rilotumumab groups (pooled) *vs* 4.2 mo in the placebo group; HR = 0.60; *P* = 0.0116]. In an exploratory analysis of this trial, MET-positivity was defined as ⩾ 25% tumor membrane staining and this MET-positive subgroup appeared to have a benefit in overall survival with rilotumumab. Meanwhile, in early phase trials of ABT-700, an anti-c-MET monoclonal antibody, and AMG 337, an oral MET kinase inhibitor, promising efficacy was shown in subgroups of patients with *MET*-amplified tumors as assessed by FISH[46,47].

Well-defined MET positivity appears to be critical for the success of MET inhibitors in GC. This may depend upon the tumor characteristics, properties of the IHC assay, and characteristics of the therapeutic agents. As suggested previously, assessing MET overexpression by detecting the extracellular domain of MET is more likely to predict the efficacy of a monoclonal antibody, such as rilotumumab[45]. However, further validation studies that are based on larger sample sizes are needed to bolster this conclusion.

Other potential biomarkers in GC

The fibroblast growth factor signaling pathway is also considered to be a potential target for the treatment of GC[39,41,48-50]. AZD4547[51] and dovitinib[52] are fibroblast growth factor receptor 2 (FGFR2) tyrosine kinase inhibitors that are currently under investigation in phase 2 trials for GC (NCT01457846 and NCT01719549). Both trials include patients with *FGFR2*-amplified GC, but use different detection methods (FISH in the AZD4547 trial and quantitative real-time PCR in the dovitinib trial). In a study that compared the result obtained using quantitative real-time PCR, IHC, and FISH in GC tissue samples, robust correlations of both quantitative real-time PCR and IHC data were shown with the FISH findings, which represents the most commonly used technique and is considered to be a standard method for FGFR2[53]. However, this correlation should be validated in future prospective clinical trials.

Olaparib, an oral small molecule inhibitor of poly (ADP-ribose) polymerase (PARP), was tested in a randomized phase 2 trial[54]. For inclusion in that study, ataxia telangiectasia mutated (ATM)-negative tumors assessed using an IHC assay were necessary considering the preclinical finding that low ATM protein expression correlates with olaparib sensitivity in GC cell lines[55]. Although progression-free survival, the primary endpoint of that study, did not differ between

the olaparib and placebo groups, overall survival was significantly improved in the olaparib group compared with the placebo group[54]. The mechanism underlying the benefit in overall survival without causing a difference in progression-free survival was unclear based on that study. However, a phase 3 trial of olaparib for GC is currently ongoing (NCT01924533) and its results will be helpful for determining whether the absence of ATM expression is a predictive biomarker for PARP inhibitors.

As ramucirumab, an anti-VEGFR-2 antibody, has been approved for the treatment of GC based on the success of the REGARD and RAINBOW trials[16,17], targeting the VEGF pathway is now considered to be a valid strategy for treating GC. However, no biomarker has been established that can predict the efficacy of ramucirumab. Further studies are urgently required to identify potential biomarkers for VEGF-targeted therapy, including ramucirumab.

CHALLENGES IN THE DEVELOPMENT OF COMPANION DIAGNOSTICS

Despite some achievements in the development of drug-companion diagnostics for GC, many challenges remain to be solved, including some that have been overlooked[56,57]. These include an inadequate understanding of the modes of action of therapeutic targets and molecules, intra- and inter-tumor heterogeneity, inadequate preclinical models for the discovery and validation of targets and biomarkers, and insufficient availability of data to select analytical platforms, methodologies, or reagents[56]. Moreover, even for established companion diagnostic tools, such as HER2 in GC, test results can be affected by preanalytical variables, including sample quality and stability, as well as the subjectivity of pathologists in assay interpretation, particularly for IHC[31,56,58]. These problems may contribute to false-positive or false-negative results that can result in unnecessary or ineffective treatments. Furthermore, this issue may be closely related to the success of biomarker-driven trials in the evaluation of therapeutics directed against novel targets.

CONCLUSION

In this era of targeted therapies, the concurrent development of companion diagnostic techniques is critical for the success of novel therapeutic agents. Additionally, the success of the development of novel drugs along with companion diagnostics largely depends upon the validity of the biomarker hypothesis, which requires a thorough molecular understanding of both tumor biology and mechanisms of drug action. Testing candidate companion diagnostic techniques in multiple phase 1 and 2 trials that incorporate biomarker analysis is also necessary, because the biomarker hypothesis for certain drugs is often derived from data obtained during the preclinical and early clinical phases of drug development[19,56].

Some of the key biological characteristics of tumors may be represented as potential biomarkers that are shared among different cancer types, such as HER2-positivity, which is a validated therapeutic target in both breast cancer and GC. Accordingly, in the future, the co-development of drugs and diagnostics could be conducted simultaneously across different cancer types. However, cancer type-specific modifications and validation will remain essential for optimizing the performance of companion diagnostic techniques.

Currently, approved companion diagnostic devices are based on the paradigm of "one biomarker, one drug", and depend on focused, low-throughput techniques, such as IHC and FISH, which have a narrow scope of biomarker evaluation and often require a relatively large amount of tissue samples[19,56,59]. Although this is not currently a critical issue in the treatment of GC, it will likely become a major problem if the need emerges to conduct various biomarker tests simultaneously for the selection of targeted agents because the amount of suitable biopsy tissues for biomarker analysis can be limited, particularly in patients who initially present with metastatic disease. Furthermore, as not all biomarker-positive patients are responsive to the corresponding drugs, so the simultaneous detection of accompanying genetic aberrations that confer resistance will also be important[59,60]. These potential challenges underscore the need for high-throughput companion diagnostic technique platforms, including next generation sequencing and mass spectrometry proteomics. These will allow us to more comprehensively assess the biological features of tumor samples. As the costs of these techniques continue to decrease, hurdles to the incorporation of high-throughput techniques in both clinical trials and daily practice are likely to be cleared, resulting in the identification of more optimal therapeutics.

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TOPIC HIGHLIGHT

2015 Advances in Gastric Cancer

Dysregulation of non-coding RNAs in gastric cancer

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Abstract

Gastric cancer (GC) is one of the most common cancers in the world and a significant threat to the health of patients, especially those from China and Japan. The prognosis for patients with late stage GC receiving the standard of care treatment, including surgery, chemotherapy and radiotherapy, remains poor. Developing novel treatment strategies, identifying new molecules for targeted therapy, and devising screening techniques to detect this cancer in its early stages are needed for GC patients. The discovery of non-coding RNAs (ncRNAs), primarily microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), helped to elucidate the mechanisms of tumorigenesis, diagnosis and treatment of GC. Recently, significant research has been conducted on non-coding RNAs and how the regulatory dysfunction of these RNAs impacts the tumorigenesis of GC. In this study, we review papers published in the last five years concerning the dysregulation of non-coding RNAs, especially miRNAs and lncRNAs, in GC. We summarize instances of aberrant expression of the ncRNAs in GC and their effect on survival-related events, including cell cycle regulation, AKT signaling, apoptosis and drug resistance. Additionally, we evaluate how ncRNA dysregulation affects the metastatic process, including the epithelial-mesenchymal transition, stem cells, transcription factor activity, and oncogene and tumor suppressor expression. Lastly, we determine how ncRNAs affect angiogenesis in the microenvironment of GC. We further discuss the use of ncRNAs as potential biomarkers for use in clinical screening, early diagnosis and prognosis of GC. At present, no ideal ncRNAs have been identified as targets for the treatment of GC.

Key words: Gastric cancer; Dysregulation; Non-coding RNA; Tumorigenesis; Biomarker

Core tip: Gastric cancer (GC) is a significant threat to the health of patients. Non-coding RNAs, primarily microRNAs and long non-coding RNAs, play important roles in gastric tumorigenesis. In this study, we review papers published in the last five years on the dysregulation of non-coding RNAs, especially microRNAs and long non-coding RNAs, in GC. We summarize how aberrant expression of the non-coding RNAs in GC affects cancer cell survival and metastasis, as well as angiogenesis within the tumor microenvironment. We additionally discuss the potential use of non-coding RNAs in the clinic as biomarkers for the diagnosis and prognosis of GC.

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INTRODUCTION

Gastric cancer (GC) is the fourth most common cancer in the world[1] and has high incidence and mortality in Asia, especially in China and Japan. The addition of widespread gastroscopy to normal practice in Japan has resulted in improved early detection rates[2]. In addition to traditional pathogenic pathways that lead to the genesis of GC, *Helicobacter pylori* (*H. pylori*) infection has also been shown to induce gastric tumor growth[3]. Previous studies of the mechanisms leading to GC hypothesized that tumorigenesis was occurring primarily through abnormal protein-protein interactions[4-6]. There has been interest in uncovering these underlying mechanisms[7,8]. Recently, significant research has been conducted on non-coding RNAs, including small non-coding RNAs and long non-coding RNAs (lncRNAs)[9-11]. Non-coding RNA regulatory dysfunction plays a significant role in the development of GC.

MicroRNAs (miRNAs) are noncoding single-stranded RNA molecules of approximately 22 nucleotides that are coded by endogenous genes and target specific mRNA molecules by forming miRNA-induced silencing complexes, resulting in mRNA degradation or hindrance of mRNA translation to functional protein. At present, miRNAs have been found to have many biological functions[12-14], including regulation of cell growth and differentiation. Thus, miRNAs play a key role in the life cycle of the cell, and their dysfunction can lead to a diseased state[15-18]. Most miRNAs are highly conserved in the genome and have a high degree of tissue specificity and temporal regulation[19-21]. These properties make miRNAs ideal biomarkers that can be detected for cancer identification. Early detection and accurate monitoring of biomarkers are crucial for treatment and positive prognosis in cancer patients, making biomarker identification particularly significant[22,23]. Recently, cell-free nucleic acids, including a population of miRNAs, have been identified in the blood of cancer patients, and their clinical relevance is attracting considerable attention[24].

LncRNAs are RNA molecules longer than 200 nucleotides that are not translated into protein. They constitute a major, although still poorly characterized, component of the human transcriptome. However, growing evidence suggests that they play an important regulatory role in many cell processes[25-27]. Nonetheless, lncRNAs remain among the least well understood of the RNA transcripts. Although previously dismissed as transcriptional "noise", several lines of evidence have suggested that lncRNAs are biologically functional[28]. LncRNAs, particularly highly conserved ones, are generally actively regulated and may function predominantly during embryonic development. Most lncRNAs evolved rapidly in terms of sequence and expression levels, but tissue specificity is often conserved[29]. It is becoming increasingly clear that many lncRNAs are deregulated in cancer, and some are functionally tied to mechanisms that may allow them to be important drivers of malignant transformation.

Protein-protein interaction networks are increasingly being employed to characterize cellular processes. These networks will have to be expanded considerably to characterize all of the possible modes of action that can occur. For example, miRNAs can silence target genes, and lncRNAs in turn can interfere with gene silencing. In contrast, lncRNA interference with target proteins can be influenced by miRNAs. For example, lncRNA-RoR is a key competing endogenous RNA that links the network of miRNAs to core transcription factors[30]. Besides protein-miRNA interactions, protein-lncRNA interactions, and micRNA-lncRNA interactions, there are many possible and uncharacterized interactions that could become dysfunctional and drive tumor development. Consequently, the development of appropriate biomarkers derived from these non-coding RNAs that reflect an individual's cancer risk is essential to reduce GC-related mortality.

DYSREGULATION OF NON-CODING RNAS IN GC

MiRNAs

MiRNAs play critical roles in physiological and pathological processes[31-33]. Using miRNA microarray technology, it has been discovered that thousands of miRNAs are dysregulated in GC compared with adjacent tissues; however, only a fraction of these were confirmed through quantitative real-time PCR. Table 1, Table 2 and Table 3 show the major miRNAs that have been confirmed by quantitative

Table 1 MicroRNAs down-regulated in gastric cancer

ncRNA	Summary of findings/ clinical relevance	q-PCR	Targets	Roles	Cases	Location	Ref.
miR-141	Reduced in metastasis positive tissues; Might be a prognostic marker and therapeutic target	√	TAZ	Proliferation, invasion and migration	36 GC *vs* paired adjacent tissues	China	[223]
miR-874	Reduced miR-874 promotes angiogenesis *via* STAT3; Might be a therapeutic target	√	STAT3	Tumor growth and angiogenesis	80 GC *vs* paired adjacent tissues	China	[187]
miR-101	Lower level parallels with EZH2 overexpression	√	EZH2	E-cadherin dysfunction	37 GC *vs* 5 normal gastric mucosa	Portugal	[224]
miR-103a	Tumor suppressor by targeting c-Myb	√	c-Myb	Proliferation, invasion and migration	80 GC *vs* paired adjacent tissues	China	[225]
miR-335	Can be silenced by promoter hypermethylation which might be a predictive epigenetic marker and a therapeutic strategy	√	RASA1	Invasion and metastasis	15 GC *vs* paired adjacent tissues	China	[52]
miR-335	Could be a therapeutic target for GC therapies and a prognostic factor	√	Bcl-w, SP1	Proliferation, invasion and metastasis	70 GC *vs* paired adjacent tissues	China	[226]
let-7a	A potential target for diagnosis and therapy	√	RAB40C	Proliferation and colony formation	27 GC *vs* paired adjacent tissues	China	[227]
miR-490-3p	Reduced miR-490-3p reactivates SMARCD1 to confer malignant phenotypes	√	SMARCD1	Growth and metastasis	14 GC *vs* 15 normal gastric tissues	Hong Kong	[228]
miR-200c/141	Reduced miRNA decreases ZEB1/2 expression and increases E-cadherin expression	√	ZEB1/2	Invasion and migration	64 GC *vs* paired adjacent tissues	China	[229]
miR-200b /c	Might be a marker of prognosis and therapeutic target	√	DNMT3A, DNMT3B, SP1	Proliferation, invasion and migration	36 GC *vs* paired adjacent tissues	China	[230]
miR-200	Down-regulated miR-200 reduced E-cadherin expression, playing a role in the carcinogenesis of EBV-associated GC	√	ZEB1, ZEB2	Cell-to-cell adhesion and migration	36 GC *vs* paired adjacent tissues (EBV-associated and EBV-negative)	Japan	[231]
miR-204-5p	Restoration of miR-204-5p might provide a therapeutic strategy for GC	√	USP47, RAB22A	Proliferation	102 GC *vs* paired adjacent tissues	China	[232]
miR-367	A key negative regulator of invasion and metastasis of GC; Might be a therapeutic target	√	Rab23	Invasion and migration	37 GC *vs* paired adjacent tissues	China	[233]
miR-328	MiR-328-mediated CD44 overexpression may associate with the carcinogenesis of GC	√	CD44v9	Survival and proliferation of metaplastic cells	54 patients underwent gastric resection without preoperative treatment	Japan	[181]
miR-328	Macrophages mediated miR-328-CD44 signaling may be a therapeutic target for gastrointestinal cancer	√	CD44	Cell growth and drug resistance	63 GC *vs* paired adjacent tissues	Japan	[234]
miR-495	A tumor suppressor and potential therapeutic target for GC peritoneal metastasis	√	PRL-3	Invasion and metastasis	20 GC *vs* 10 normal gastric tissues	China	[53]
miR-551a	A tumor suppressor targeting PRL-3 oncogene to inhibit GC cell migration and invasion	√	PRL-3	Invasion and migration	30 malignant *vs* 4 normal gastric tissues	China	[235]
miR-133b	A potential diagnostic marker and therapeutic target	√	FSCN1	Proliferation, invasion and migration	100 GC *vs* paired adjacent tissues	China	[236]
miR-542-3p	A tumor suppressor and a potential therapeutic target	√	AEG-1	Cell growth	22 GC *vs* paired adjacent tissues	China	[112]
miR-126	Suppresses tumor growth and angiogenesis through targeting VEGF-A; A potential therapeutic target	√	VEGF-A	Tumori-genicity and angiogenesis	68 GC *vs* paired adjacent tissues	China	[119]
miR-126	May function as a tumor suppressor in GC	√	Crk	Proliferation, cell cycle, apoptosis, invasion and migration	60 GC *vs* paired adjacent tissues	China	[237]
miR-29s	Increasing the expression of miR-29s may be a therapeutic strategy for GC	√	AKT2	Invasion	20 GC *vs* paired adjacent tissues	China	[120]

miR-29c	Reduced miR-29c expression is an early event in GC development; Potential diagnostic and therapeutic biomarkers	√	ITGB1	Proliferation, adhesion, invasion and migration	274 GC *vs* paired adjacent tissues	South Korea, Japan, United States	[215]
miR-29 family	Might be potential prognostic markers and therapeutic targets	√	CCND2, MMP-2	Proliferation apoptosis and invasion	115 GC *vs* paired adjacent tissues	China	[238]
miR-29c	Might be a tumor suppressor	√	RCC2	Proliferation and colony formation	12 GC *vs* paired adjacent tissues	Japan	[239]
miR-193b	Might be a potential prognostic marker	√	Unknown	Differentiation and survival	48 GC *vs* paired adjacent tissues	China	[240]
miR-203	Might be a therapeutic target for *H. pylori* infection induced GC	√	CASK	Proliferation and invasion	50 pairs of *H. pylori* positive and negative gastric tissues	China	[241]
miR-210	Epigenetic silencing of miR-210 involves in chronic *H. pylori* infection associated GC	√	STMN1, DIMT1	Proliferation	20 GC *vs* paired adjacent tissues	Japan	[242]
miR-34 family	Plays a role in the control of GC development	√	Yin Yang 1	Growth, colony formation, migration, invasion, and tumorsphere formation	32 GC *vs* paired adjacent tissues	Taiwan	[243]
miR-34b and miR-129-3p	Down-regulated by hypermethylation of upstream CpG islands indicating a poor clinical outcome	√	Unknown	Unknown	72 GC *vs* paired adjacent tissues	Taiwan	[244]
miR-24	A novel tumor suppressor and a potential therapeutic target	√	RegⅣ	Proliferation, invasion and migration	63 GC *vs* paired adjacent tissues	China	[245]
miR-185	Regulating the sensitivity of GC to chemotherapy	√	ARC	Chemotherapeutic sensitivity	25 GC *vs* paired adjacent tissues	China	[246]
miR-1207-5p and miR-1266	hTERT suppressors in GC and potential therapeutic targets	√	hTERT	Cell growth, cell cycle and invasion	58 GC *vs* adjacent tissues	China	[108]
miR-365	Playing a role in tumorigenesis; A potential therapeutic target	√	Cyclin D1, cdc25A	Proliferation and colony formation	127 GC *vs* paired adjacent tissues	China	[105]
miR-760	A potential prognostic predictor and therapeutic target	√	Histone mRNA	Unknown	53 bone marrow samples from stage Ⅳ patients *vs* 52 stage Ⅰ patients; 22 stage Ⅳ GC *vs* 29 stage Ⅰ GS tissues	Japan	[247]
miR-143/145	DDX6 contributes to the control of NCR143/145 RNA stability in P-bodies and post-transcriptionally regulated miR-143/145 expression	√	Unknown	Cell survival, proliferation and malignant transformation	14 GC tissues *vs* paired adjacent tissues	Japan	[84]
miR-206	A potential tumor suppressor and therapeutic target	√	CyclinD2	Proliferation, cell cycle and tumor growth	30 primary GC *vs* paired distant tissues	China	[248]
miR-204	A potential target for preventive and therapeutic strategies	√	Bcl-2	Migration, colony forming and chemotherapy resistance	92 gastric tumor specimens *vs* paired adjacent tissues	Italy	[249]
miR-124	A tumor suppressor; Play a role in miRNA-mediated SPHK1 expression	√	SPHK1	Proliferation and tumourigenicity	20 GC *vs* paired adjacent tissues	China	[117]
miR-409-3p	A tumor suppressor involving the direct targeting and inhibition of PHF10	√	PHF10	Proliferation and apoptosis	67 GC *vs* paired adjacent tissues	China	[250]
miR-409-3p	Suppresses GC invasion and metastasis by directly targeting RDX; Reduced miR-409-3p is prone to lymph node metastasis	√	RDX	Invasion and migration	90 GC *vs* paired adjacent tissues	China	[251]
miR-148a	Reduced miR-148a contributes to GC lymph node-metastasis and progression; A potential therapeutic target for GC metastasis	√	ROCK1	Invasion, migration and metastasis	90 GC *vs* paired normal tissues	China	[252]
miR-148b	A potential biomarker and therapeutic target	√	CCKBR	Proliferation and tumorigenicity	106 GC *vs* paired adjacent tissues	China	[253]
miR-449	A member of the miR-34 family playing an important role in GC	√	GMNN, MET, CCNE2, SIRT1	Cell cycle, proliferation and induce senescence	10 GC *vs* paired adjacent tissues	Denmark	[254]
miR-486	A tumor-suppressor; Associated with the direct targeting and inhibition of OLFM4	√	OLFM4	Proliferation, invasion and migration	29 GC *vs* paired adjacent tissues	Singapore	[255]

miR-142-5p	A potential predictor of progression and predict recurrence risk for GC	√	MAPK, Wnt, VEGF	Recurrence risk related	65 GC samples	China	[256]
miR-125a-5p	Reduced miR-125a-5p is associated with enhanced malignant potential; A potential prognostic marker	√	ERBB2	Proliferation	87 GC samples	Japan	[257]
miR-516a-3p	An anti-metastamir with therapeutic potential in blocking metastatic dissemination of GC	√	SULF1	Proliferation, invasion and migration	8 normal stomach tissues, 12 GC tissues from the patients with peritoneal dissemination and 12 GC tissues without peritoneal dissemination	Japan	[258]
miR-181c	Silenced through methylation playing important roles in gastric carcinogenesis	√	NOTCH4, and KRAS	Proliferation	16 GC surgical specimens *vs* paired non-cancerous counterparts	Japan	[259]
miR-212	Reduced miR-212 may be related to gastric carcinogenesis	√	MECP2	Proliferation	11 GC *vs* paired adjacent tissues	Japan	[260]
miR-338-3p	MiR-338-3p inhibits the EMT progression in GC cells by targeting ZEB2 and MACC1/Met/Akt pathway	√	ZEB2, MACC1	Invasion and migration	20 GC *vs* paired adjacent tissues	China	[261]
miR-217	A potential prognostic marker; miR-217-EZH2 axis may be a potential therapeutic target	√	EZH2	Proliferation, invasion and migration	83 GC tissues *vs* adjacent tissues	China, United States	[262]
miR-15a and miR-16-1	MiR-15a and miR-16-1 have inhibitory effect providing a therapeutic potential in GC	√	YAP1	Proliferation, colony formation, invasion and migration	60 GC *vs* paired adjacent tissues	Hong Kong	[263]

GC: Gastric cancer.

reverse transcription-PCR (qRT-PCR) and found to be dysregulated in GC tissues since 2010. Many of these miRNAs were demonstrated to act as tumor promoters or suppressors by regulating the expression levels of their target mRNAs in GC cells. However, the mechanisms that control miRNA regulation are as of yet unknown. Below, we have listed some mechanisms linked to altered miRNA expression in GC cells.

Multiple miRNAs were found to be dysregulated in *H. pylori*-positive GC tissues compared with *H. pylori*-negative GC tissues. It was reported that a total of 219 of the 3523 measured miRNAs showed a 2-fold up- or down-regulation in *H. pylori*-positive GC tissues compared with *H. pylori*-negative GC tissues[34]. Further studies revealed three miRNAs (miR-99b-3p, miR-564, and miR-638) that were significantly up-regulated in three *H. pylori*-positive GC samples, while four miRNAs (miR-204-5p, miR-338-5p, miR-375, and miR-548c-3p) were significantly down-regulated in all eight *H. pylori*-positive GC samples. In addition, the levels of miR-223 and miR-222 were up-regulated while miR-375 and miR-320 were down-regulated in *H. pylori*-infected gastric mucosa[35-38]. MiR-146a was up-regulated in *H. pylori*-infected human gastric epithelial cells and has been shown to decrease the inflammatory response induced by *H. pylori* partially through reducing the level of PTGS2[39]. Further work revealed that miR-146a could enhance apoptosis in GC cells, and there was a positive correlation between miR-146a level and the apoptosis rate in *H. pylori*-positive GC tissues. The mechanism by which these miRNAs become dysregulated is still unclear and requires further investigation, but NF-κB, a key transcription factor in the development of *H. pylori*-induced chronic inflammation, may play a critical role in this process. Accordingly, the level of miR-200 was increased in *H. pylori*-infected GC cells, which was driven by a functional NF-κB binding site in the promoter of the miR-200b-200a-429 cluster. This strongly suggests that NF-κB plays an important role in the direct regulation of miR-200 transcription[40]. Increased expression of miR-200 may be a response to the unalterable loss of the epithelial phenotype of GC cells induced by *H. pylori*. MiR-155 was up-regulated by *H. pylori* both *in vitro* and *in vivo*, and this induction was NF-κB dependent[41]. In addition, *H. pylori* could also induce the expression of miR-155 in T cells in a cAMP-Foxp3-dependent manner[42] and in macrophages in a T4SS-dependent manner[43]. MiR-155 was proven to be necessary for Th17/Th1 differentiation and the induction of chronic gastritis in a mouse model infected with *H. pylori*[44]. Furthermore, increased levels of miR-155 suppressed the production of IL-8 induced by *H. pylori* in gastric epithelial cells[41] by regulating the expression of MyD88[45]. IL-6 is a pro-inflammatory cytokine negatively regulated by miR-155 and miR-146b in *H. pylori*(cagA+)-induced gastroduodenal ulcers[46]. Let-7b was found to be involved in the activation of NF-κB in response to *H. pylori* induced inflammation and immune responses[47]. Let-7b was down-regulated in *H. pylori*-infected gastric epithelial cell lines and the forced overexpression of let-7b inhibited the activation of NF-κB by suppressing the level of TLR4 in these cells. These results demonstrate that let-7b is a negative regulator of NF-κB and that this may be the reason for let-7b down-regulation in *H. pylori*-infected gastric epithelial cells. The levels of several pro-inflammatory cytokines in *H. pylori* induced chronic inflammation, including IL-1β, IL-6, IL-8, and TNF-α, were found to be correlated

Table 2 MicroRNAs up-regulated in gastric cancer

ncRNA	Summary of findings/clinical relevance	q-PCR	Targets	Roles	Cases	Location	Ref.
miR-23a/b	Implicated in the progression of GC. A potential prognosis marker	√	Unknown	Unknown	160 GC *vs* adjacent tissues	China	[264]
miR-500	Highly correlated with malignant progression and poor survival of GC	√	CYLD, TAX1BP1, OTUD7B	Proliferation, survival and tumorigenicity	10 GC *vs* adjacent tissues	China	[137]
miR-374a	A promising therapeutic target	√	SRCIN1	Proliferation, tumor growth migration and invasion	18 GC tissues *vs* adjacent tissues	China	[265]
miR-199a-3p	A tumor promoter in GC targeting and inhibition of ZHX1; A potential target for GC prevention and therapy	√	ZHX1	Proliferation and apoptosis	52 GC *vs* adjacent tissues	China	[266]
miR-18a	A potential marker for risk stratification in the management of GC patients	√	PIAS3, STAT3	Unknown	82 patients with GC and 65 healthy controls (plasma)	China	[204]
miR-196a	A potential prognostic marker in GC	√	Unknown	Differentiation and survival	48 GC *vs* adjacent tissues	China	[240]
miR-223, miR-16, miR-100	Up-regulated in serum implicates their potential diagnostic value; Significantly elevated expression of the three miRNAs in advanced GC patients suggests their availability in cancer staging	√	PIAS3	Unknown	50 GC patients and 47 healthy controls (serum)	China	[202]
miR-135a-5p	Play a role in miRNA-135a-5p-AP-2α-BCL-2 pathway providing therapeutic potential for GC and solution for insensitivity of GC to chemotherapy	√	AP-2α	Cell resistance to apoptosis, sensitivity to adriamycin	20 GC *vs* adjacent tissues	China	[267]
miR-199a-5p	SRF/miR-199a-5p/E-cadherin pathway promotes GC EMT and metastasis; A potential therapeutic target or biomarker for GC progression	√	E-cadherin	Adhesion, invasion, and metastasis	7 GC *vs* pairs adjacent tissues	China	[268]
miR-25	A potential biomarker for the prognosis of GC	√	ERBB2, 1(TOB1)	Migration, invasion and proliferation	33 GC *vs* paired adjacent tissues	China	[269]
miR-942	A potential drug response biomarker and therapeutic target for TRAIL resistant tumors	√	ISG12a	Apoptosis	28 GC tissues	China	[134]
miR-196a/b	A potential therapeutic target in suppressing GC metastasis	√	Radixin	Metastasis	109 GC *vs* paired adjacent tissues	Taiwan	[172]
miR-19a/b	A member of miR-19a/b facilitating GC cell migration, invasion and metastasis, implicating a novel mechanism for the malignant phenotypes of GC	√	MXD1	Migration and invasion	141 GC *vs* paired adjacent tissues	China	[173]
miR-423-5p	A potential therapeutic target	√	TFF1	Proliferation and invasion	15 GC *vs* paired adjacent tissues	China	[270]
miR-183-96-182 cluster	A novel role for GSK3β in the regulation of miR-183-96-182 biogenesis through β-catenin/TCF/ LEF-1 pathway in GC	√	FoxO1	Proliferation and migration	8 GC *vs* paired adjacent tissues	United States	[83]
miR-215	Influencing cell proliferation by targeting RB1	√	RB1	Proliferation	51 GC *vs* paired adjacent tissues	China	[271]
miR-17-92 cluster	Cluster including miR-19b, miR-20a and miR-92a associates with the development of GC stem cells; and miR-92a as a potential predictive prognostic marker for miR-92a in GC	√	E2F1, HIPK1	Self-renewal and proliferation	97 GC specimens	China	[272]
miR-296-5p	MiR-296-5p-CDX1-ERK1/2 axis play a role in gastric tumorigenesis; A potential therapeutic target	√	CDX1	Proliferation	16 GC *vs* paired adjacent tissues	China	[273]
miR-181a	Associated with increased risk and poor survival of GC	√	MTMR3	Unknown	50 GC *vs* paired adjacent tissues	China	[274]
miR-196a	Contributing to gastric carcinogenesis; A potential therapeutic target and prognostic factor	√	p27	Proliferation, apoptosis and tumorigenesis	36 GC *vs* paired adjacent tissues	China	[275]
miR-196b	Transcriptionally regulated by ETS2; A potential diagnostic marker and therapeutic target	√	AnnexinA1, HOXB8	Migration and invasion	63 GC *vs* paired adjacent tissues	Taiwan	[276]
miR-378	Up-regulated in serum while down-regulated in GC tissues. A potential serum biomarker in GC detection	√	Unknown	Unknown	4 GC *vs* paired adjacent tissues, 40 GC serum samples *vs* 41 healthy controls	China	[277]

miR-370	Associated with GC progression by targeting TGFβ-RII	√	TGFβ-RII	Migration	33 GC *vs* adjacent tissues	Taiwan	[278]
miR-192 miR-215	Exerting cell growth and migration-promoting effects	√	ALCAM	Migration, invasion, proliferation, cell cycle and apoptosis	31 non-neoplastic stomach tissues and 25 GC tissues	United States	[279]
miR-200b	A potential diagnostic and prognostic biomarker; A potential therapeutic target for peritoneal dissemination	√	Unknown	Migration and invasion	173 GC *vs* paired normal gastric epithelium tissues	Japan	[280]

GC: Gastric cancer.

Table 3 MicroRNAs up-regulated or down-regulated in gastric cancer

ncRNA	Summary of findings/clinical relevance	q-PCR	Targets	Roles	Cases	Location	Ref.
miR-183 ↑	A potential biomarker for GC progression and therapeutic target	√	PDCD4	Proliferation, migration, invasion, and apoptosis	80 GC *vs* 20 non-tumorous gastric mucosa tissues	China	[131]
miR-183 ↓	A tumor suppressor partially through regulation of Ezrin; A potential therapeutic target	√	Ezrin	Invasion	52 pairs of paraffin-embedded GC and adjacent tissues; 5 fresh tissues samples from three patients	China	[281]
miR-146a ↑	A key factor in the regulation of NF-κB activity	√	CARD10, COPS8	Inhibits NF-κB activation	37 GC *vs* paired adjacent tissues	Denmark	[282]
miR-146a/b ↓	MiR-146a/b/UHRF1 axis associates with the GC metastasis; A potential therapeutic target in blocking GC metastasis	√	UHRF1	Invasion and metastasis	15 primary GC tissues compared with matched adjacent normal tissues	China	[180]
miR-146a ↓	MiR-146a/WASF2 axis may associate with the migration and invasion of GC cells; A potential therapeutic target	√	WASF2	Invasion and metastasis	20 GC *vs* paired adjacent tissues	China	[283]
miR-146a ↓	Targeting EGFR and IRAK1; A potential prognostic factor	√	EGFR, IRAK1	Invasion and metastasis	90 GC *vs* paired adjacent tissues	Japan	[179]
miR-9 ↑	Targeting and suppressing CDX2 expression promote GC cell proliferation	√	CDX2	Proliferation	27 GC tissues	Japan	[284]
miR-9 ↓	Ectopic expression of miR-9 inhibits the proliferation, migration and invasion of GC cells	√	MMP2, MMP9, Twist, N-cadherin	Invasion and metastasis	72 GC *vs* adjacent tissues	Taiwan	[285]
miR-9 ↓	A tumor suppressor targeting NF-κB1	√	NF-κB1	Proliferation	9 GC *vs* paired adjacent tissues	China	[286]
miR-375 ↑	A predictor of GC; progression and recurrence risk for GC patients	√	P53, MAPK, Wnt, VEGF	High frequency recurrence and poor survival	34 frozen fresh tissues and 38 paraffin-embedded tissues	China	[256]
miR-375 ↓	A tumor suppressor; Playing a role in gastric tumorigenesis	√	JAK2	Proliferation	48 GC *vs* paired adjacent tissues	China	[287]
miR-375 ↓	A tumor suppressor	√	PDK1, 14-3-3zeta	Apoptosis, proliferation	22 samples from GC and 5 normal control tissues	Japan	[288]
miR-218-5p ↑	MiR-218-5p targets and suppresses TFF1 and influences the progression of GC in an Erk1/2-dependent manner; A potential therapeutic target	√	TFF1	Proliferation	42 GC *vs* paired adjacent tissues	China	[289]
miR-218 ↓	Disruption of Slit-miR-218-Robo1 regulatory circuit may contribute to GC metastasis. A potential therapeutic target in blocking GC metastasis	√	Robo1	Invasion and metastasis	40 GC *vs* paired adjacent tissues	China	[290]

GC: Gastric cancer.

with miRNA expression[48]. This evidence suggests the possibility that chronic inflammation mediated by pro-inflammatory cytokines plays a role in regulating the expression of miRNAs in *H. pylori*-infected GC, though the mechanism by which this might occur remains unknown.

Accumulated evidence shows that DNA methylation of miRNA promoter sites is a critical mechanism for miRNA dysregulation in tumors, including GC. Investigation of the methylation frequency of 9 miRNA CpG islands in human gastric samples, including gastritis, GC and normal tissues, revealed that methylation frequency was increased in 5 CpG islands (miR-9-1, miR-9-3, miR-137, miR-34b, and

miR-210) and decreased in 1 CpG island (miR-200b) during gastric carcinogenesis[49]. Furthermore, the methylation of those 6 miRNA CpG islands in cells significantly suppressed the expression of the corresponding miRNAs. MiR-137, which acts as a tumor suppressor, was found to be down-regulated in GC[50] through methylation of a CpG island in its promoter, and an analysis of clinical samples showed that methylation of miR-137 occurred frequently in GC and played a role in gastric carcinogenesis[51]. Methylation-induced miRNA silencing in GC was also observed with miR-335[52], miR-495[53], miR-9[54], miR-10b[55], miR-219-2-3p[56], miR-212[57], miR-941 and miR-1247[58]. Aberrant expression of these miRNAs and consequent regulation of their corresponding targets resulted in changes in GC cell growth, invasion and migration[52-56,58]. Furthermore, the suppression of miRNA expression was restored after treatment with 5-aza-2′-deoxycytidine, an agent designed to reduce the degree of methylation in GC cells at specific miRNA sites. MiR-129-5p is a multi-drug resistance-related miRNA that becomes down-regulated in the drug-resistant cell line SGC7901/VCR *via* methylation, as evidenced by a restoration of miR-129-5p levels upon 5-aza-2′-deoxycytidine treatment in these cells[59]. MiR-34c-5p also negatively regulates paclitaxel resistance of GC cells and is down-regulated by a methylation of CpG islands that are near the miR-34 promoter[60]. These experiments show that methylation can regulate the levels of miRNAs. Conversely, miRNAs can regulate DNA methylation by targeting DNA methyltransferases (DNMTs). Previous experiments have shown that miR-148a modulated the expression of DNMT1 and caused the overexpression of miR-148a, and miR-148a reduced the methylation of the RUNX3 promoter, culminating in increased RUNX3 mRNA and protein in GC cells[61].

There are other regulatory elements that can induce aberrant expression of miRNAs. For example, TGF-β, a critical cytokine in cancer, can regulate miRNA expression. Specifically, this cytokine can up-regulate miR-155[62] and miR-181a[63] in hepatocyte cell lines and down-regulate miR-203 through direct binding to the promoter[64]. TGF-β1 treatment has been shown to alter miRNA expression in GC cells, causing the up-regulation of 3 miRNAs and down-regulation of 3 miRNAs[65]. TGF-β1 regulate gene expression in a Smad-dependent or -independent manner. However, the role that TGF-β1 plays in regulating the expression of miRNAs in GC is not often reported and the mechanism still requires elucidation. In addition, certain oncogenes play a critical role in the dysregulation of miRNAs in cancer. For example, miR-29b was inhibited by c-myc in non-small cell lung cancer[66] possibly through the regulation of Drosha[67]. P53 has also been reported to modulate the expression of miR-34a[68]; however, this protein has not been found in GC, and the role it plays in miRNA regulation is still uncertain. Hypoxia is another modulator of miRNA expression and functions through HIF-1α. MiR-382 was demonstrated to be induced by HIF-1α in GC cells under a hypoxic stress[69], and this phenomenon was also observed in ovarian carcinoma[70], lung cancer[71] and other cancer cell lines[72-74]. The expression profile of miRNAs also changes in GC when the cells undergo treatment with anti-tumor drugs. Treatment of GC patients with cisplatin and docetaxel significantly increased the expression of members of the miR-29 family, causing an inhibition of GC metastasis[75]. Moreover, some miRNAs that are modulated by anti-tumor drugs, such as miR-508-5p[76], miR-1271[77], and miR-503[78], might participate in the development of drug resistance in GC cells[79-82]. MiRNA regulation also occurs at the protein level in GC cell lines. For example, GSK3β, a critical protein kinase, suppresses the expression of the miRNA-183-96-182 cluster, resulting in a reduction of miR-96, miR-182 and miR-183 levels in GC cells[83]. Another protein, DDX6, suppresses the expression of the miR-143/145 cluster post-transcriptionally in GC cells[84].

LncRNAs

Dysregulation of lncRNAs is involved in tumorigenesis[85], but the underlying mechanisms remain elusive. Here, we describe some recent published data linked to the mechanisms of dysregulation of lncRNAs in GC.

PVT1 expression is increased in GC tissues and cells, and the knockdown of PVT1 inhibits GC cell proliferation and lymph node invasion[7,86]. PVT1 shows potential as a novel therapeutic target for patients who would otherwise have a poor prognosis. In addition, HOTAIR was found to be critically involved in the function of GC cells and has an inverse relationship with PCBP-1 in both expression level and function. Accordingly, PCBP1 was confirmed to be an inhibitor of GC pathogenesis. SiRNA-mediated knockdown of HOTAIR in GC cells significantly inhibited cell proliferation, migration and invasion. Additionally, the impact of HOTAIR on apoptosis, cell proliferation and cell cycle regulation was investigated to dissect the carcinogenesis of GC[87,88]. In addition to these findings, HOTAIR is a target of miR-331-3p and miR-124, and therefore, it may act as a competitive endogenous RNA for the targets of those miRNAs[89].

C-Myc induces lncRNA H19 expression, with the expression of lncRNA H19 positively correlating with the c-Myc levels in 80 GC samples[90]. Overexpression of lncRNA H19 directly promotes ISM1 expression and indirectly promotes miR-675 expression in GC. An inverse relationship was also revealed between the expression of RUNX1 and lncRNA H19/miR-675 in GC tissues and cell lines. Overexpression of lncRNA H19 was shown to promote tumorigenic features of GC including proliferation, migration, invasion and metastasis[25,91]. In addition, MALAT1 and MALAT2 were

aberrantly highly expressed in gastric cell lines and tissues, and MALAT1 can mediate the overexpression of SF2/ASF in the nucleolus. Therefore, MALAT1 may function as a promoter of GC cell proliferation through the regulation of SF2/ASF[92]. Overexpression of MALAT2 in GC cells increased the migration of GC cells and induced the epithelial-mesenchymal transition (EMT) through an MAP kinase pathway[93].

TUSC7 is a p53-regulated tumor suppressor that acts in part by repressing miR-23b. It has been shown that TUSC7 expression suppressed tumor cell growth *in vitro* and *in vivo*[94]. In addition, the expression of lncRNAs LET, FENDRR, FER1L4 and HMlincRNA717 was markedly down-regulated in tumor tissues compared with adjacent non-tumor tissues. These decreases in specific lncRNA expression were correlated with deeper tumor invasion, lymph node metastasis, distant metastasis, and higher TNM stages[95-98]. However, FENDRR overexpression suppressed invasion and migration by down-regulating FN1 and MMP2/MMP9 expression in GC cells[96]. The lncRNA GAS5 was demonstrated to decrease GC cell proliferation partly *via* regulating E2F1 and P21 expression and to induce apoptosis[99]. Ectopic expression of lncRNA MEG3 was able to inhibit cell proliferation, promote cell apoptosis, and modulate p53 expression in GC cell lines, however, its expression level was significantly correlated with TNM stage, depth of invasion, and tumor size[100]. Overexpression of the lncRNA LEIGC was able to suppress tumor growth and cell proliferation and to enhance the sensitivity of GC cells to 5-fluorouracil (5-FU), whereas knockdown of LEIGC had the opposite effect. It was further demonstrated that LEIGC functions by inhibiting the EMT in GC[101].

FUNCTION

Survival

MiRNAs in cell cycle regulation: MiRNAs can regulate cell growth by influencing cell cycle-related gene expression. MiR-101 functions as a suppressor in *H. pylori*-infected GC. The ectopic expression of miR-101 results in the down-regulation of c-myc, CDK2, CDK4, CDK6, CCND2, CCND3 and CCNE2 and the up-regulation of p14, p16, p21 and p27. These changes culminate in the induction of G1-phase cell cycle arrest in GC cells, leading to an inhibition of cell growth and colony formation[102]. MiR-137 suppresses GC cell proliferation both *in vitro* and *in vivo*, through the induction of a G1/S arrest by targeting CDK6[103]. MiR-520d-3p down-regulates c-myc and CyclinD1 expression in GC cells and suppresses cell growth by binding to the 3′ untranslated region (UTR) of EphA2 mRNA[104]. MiR-365 expression is reduced at the transcriptional level in GC tissue *via* AKT signaling in a p53-dependent manner. Overexpression of miR-365 suppresses GC cell proliferation both *in vitro* and *in vivo* through direct binding to the 3′UTR of Cyclin D1 and cdc25A mRNAs[105]. Some miRNAs, including miR-300, are involved in regulating cell cycle arrest caused by ionizing radiation such as X-rays, indicating that this miRNA may play a role in regulating the GC cell cycle[106]. The MiR-191/425 cluster was found to be overexpressed in GC tissue. A loss-of-function assay indicated that the cluster has roles in cell cycle regulation, although the mechanism is unknown[107]. MiR-1207-5p and miR-1266 are both reported to reduce the expression of hTERT, resulting in G1/S cell cycle arrest and reduction of GC cell growth both *in vitro* and *in vivo*[108]. MiR-212 inhibited GC cell growth by directly reducing RBP2 expression and up-regulating critical cell cycle related proteins such as p21 and p27[109]. MiR-17-5p/20a acted as an oncogene in GC cells by directly targeting p21 and TP53INP1, which have negative roles in the cell cycle and promote GC cell growth when inhibited[110]. Some miRNAs, such as miR-101, miR-137, miR-520d-3p and miR-17-5p/20a, directly bind to the 3′UTR of cell cycle related mRNAs and reduce their expression, resulting in progression or arrest of the cell cycle[35,102,103,110]. Other miRNAs, such as miR-300, miR-191/425 cluster, miR-1207-5p, miR-1266 and miR-212[106-109], indirectly modulate cell cycle related protein levels by regulating the expression of upstream target genes, but it is unclear how these miRNAs affect the cell cycle. Changing a single factor may cause a series of diversifications of the signaling network in cell cycle activity, and the mechanisms by which this occurs still require further research and exploration.

Regulation of AKT pathway by miRNAs: The AKT pathway is dysfunctional and hyperactive in many human cancers, including GC, and plays an important role in cell survival. MiRNAs can regulate cell survival through activation or inactivation of the AKT pathway through their targets in GC. For example, miR-1274a was found to be up-regulated in GC tissue as well as in GC cell lines such as HGC27, MGC803, SGC-7901 and AGS. Interestingly, miR-1274a inhibits FOXO4 protein expression in HGC27 and MGC803 with no effect on mRNA level. A dual-luciferase reporter assay confirmed that FOXO4, which functions as an inhibitor of the PI3K/AKT pathway, was directly modulated by miR-1274a in GC cells. MiR-1274a therefore activates the PI3K/AKT pathway through inhibition of FOXO4 expression in GC cells, resulting in enhanced cell proliferation and migration. GC xenograft mouse models also indicate that miR-1274a overexpression in GC cells can promote tumorigenesis[111]. While miR-542-3p is normally expressed at a low level in GC tissues and cells, overexpression of miR-542-3p can potentially markedly inhibit the activation of the AKT pathway and reduce cell growth by directly binding to the 3′UTR of AEG-1[112]. MiR-137 could inhibit the activation of the AKT pathway through its target gene Cox-2 and suppress GC cell growth both *in vitro* and *in vivo*[50]. MiR-34a[113], miR-338[114,115], miR-21[116], miR-124[117], miR-10b[118] and other miRNAs can

also regulate AKT pathway activity through similar gene targeting. Overexpression of miR-126, which normally acts as a suppressor of angiogenesis in GC, also inhibited cell growth by reducing the activation of the AKT pathway[119]. Similarly, hypoxia-induced miR-382 expression reduced the level of PTEN in GC cells, causing an inhibition of AKT pathway. *In vivo*, down-regulation of miR-382 caused a reduction of tumor growth and reduced microvessel density[69]. The miR-29 family contains three miRNAs with identical seed sequences: miR-29a, miR-29b, miR-29c. This family was reported to reduce the expression of AKT2, a key member of AKT pathway, by directly targeting its 3'UTR in GC cell lines HGC27 and MGC803. Clinical GC tissue analysis also illustrated that the expression of miR-29 and AKT2 has a negative correlation. Finally, ectopic expression of miR-29 induced a suppression of the AKT pathway in GC cells[120]. In addition to the miR-29 family, let-7b/g also directly binds to the 3'UTR of AKT2 and subsequently results in an inhibition of AKT pathway activity in GC[121].

MiRNAs in apoptosis: Inducing or inhibiting cell apoptosis is also an important function of miRNAs in GC cells, making them important regulators of tumor suppressors and oncogenes. Generally, miRNAs that are down-regulated in GC tissues and cells have tumor suppressive functions, and some, including miR-449a[122], miR-133a[123,124], miR-224[125], miR-338[114,115,126], miR-143[127], miR-874[128] and others, are capable of inducing cell apoptosis in GC cells. Overexpression of these miRNAs induces a suppression of cell proliferation in GC cells concurrent with other effects such as cell cycle arrest, and suppression of invasion and metastasis. Tumor suppressive miRNAs have complex functions in GC cells through their known targets or potentially other pathways and may be useful targets for GC therapy. Because miR-449a acts as a tumor suppressor by directly targeting bcl-2, which is known for its anti-apoptotic function, the overexpression of miR-449a in GC cells enhances cell apoptosis and results in G1/G0 arrest[129,130].

MiRNAs can also act as oncogenes to promote tumor growth and are referred to as oncomirs. MiR-183 is an oncomir that is up-regulated in GC tissues, and its increased expression level is associated with high clinical stage, enhanced invasion, and lymph node metastasis. Moreover, overexpression of miR-183 in GC cells reduces the rate of apoptosis by affecting the expression of PDCD4[131]. Other miRNAs, such as miR-645[132], miR-181a[133], and miR-942[134], can act as oncomirs by inhibiting cell apoptosis and promoting tumor growth.

NF-κB signaling has been shown to interact with miRNAs in GC cells. IL-1β activates NF-κB in GC cells, and the activated NF-κB directly binds to the promoter of miR-425 to enhance its transcription. Up-regulated miR-425 promotes GC cell proliferation and helps the cells resist apoptosis induced by cisplatin through direct targeting of the 3'UTR of PTEN mRNA[135]. Alternatively, some miRNAs, such as miR-362, are capable of activating the NF-κB signaling pathway in GC cells[136]. MiR-362 activates the NF-κB signaling pathway by reducing the expression of the tumor suppressor CYLD, which is its direct target in GC cells. Similar to previously mentioned miRNAs, the activation of NF-κB through overexpression of miR-362 inhibited the apoptosis induced by cisplatin and promoted GC cell proliferation. MiR-500 also activates NF-κB by suppressing the expression of CYLD, OTUD7B and TAX1BP1, which are all negative regulators of NF-κB signaling. Overexpression of miR-500 leads to activation of NF-κB signaling in GC cells, and promotes resistance to apoptosis, resulting in cell proliferation and high tumorigenicity *in vivo*[137].

MiRNAs in drug resistance: Tumor resistance to chemotherapeutic drugs has become increasingly problematic in GC. Recent findings show that miRNAs affect the sensitivity of cancer cells to chemical drugs in GC by regulating the expression of target genes. Using an miRNA expression profiling chip, it was reported that there was a significant dysregulation of miRNAs in the drug resistant sublines SGC-7901/VCR and SGC-7901/ADR compared with the parental SGC-7901 line. Quantitative RT-PCR analysis demonstrated that miR-99b-5p, let-7e-5p, miR-125a-5p, miR-181a-5p and miR-100-5p were significantly down-regulated, and miR-1273g-3p, miR-378a-5p and miR-425-5p were up-regulated in drug resistant sublines[138]. Up-regulated miRNAs in drug resistant sublines prevent cancer cell death induced by chemotherapeutics, while down-regulated miRNAs promote cancer cell death during chemotherapeutic treatment.

MiR-106a was found to be up-regulated in the drug resistant sublines SGC-7901/VCR and SGC-7901/ADR. Overexpression of miR-106a was able to reduce the sensitivity of SGC-7901 to anticancer drugs and inhibit cell apoptosis. Conversely, inhibition of miR-106a in SGC-7901/VCR enhanced the sensitivity of SGC-7901/ VCR to chemotherapeutics and decreased their IC_{50} dose[81]. MiR-21 was up-regulated in SGC7901/DDP, a cisplatin resistant cell line and may be partially responsible for resistance of GC cells to cisplatin[80]. MiR-19a/b has also been shown to regulate the resistance of GC cells to anticancer drugs and was found to be up-regulated in MDR cell lines. Furthermore, an increase in miR-19a/b levels reduces the sensitivity of GC cells to drugs by accelerating ADR efflux[79].

In contrast, miR-185 was down-regulated in GC tissues. GC cells with significant overexpression of miR-185 were significantly more sensitive to apoptosis induced by low doses of chemotherapeutic agents compared with their negative controls, a finding which was confirmed in a nude mouse model. Furthermore, reduction of endogenous miR-185 expression in GC cells inhibits cell apoptosis induced by high-dose

chemotherapeutic agents[139]. MiR-218, on the other hand, could increase the sensitivity of GC cells to cisplatin and inhibit cell growth[140], and miR-1271 was found to be down-regulated in the cisplatin resistant cell line SGC7901/DDP. Overexpression of miR-1271 enhanced the response of SGC7901/DDP cells to cisplatin[77]. Finally, miR-200c was also reported to regulate drug resistance in GC cells[141].

Accumulating evidence indicates that miRNAs play an important role in the resistance of cancer cells to chemotherapy treatment; however, the mechanism by which this occurs remains poorly understood. However, the ability of the previously mentioned miRNAs to directly affect drug resistance in tumor lines reveals novel targets for improving the efficacy of chemotherapy in the future. Therefore, chemotherapy in combination with gene therapy might be a new avenue for cancer treatment going forward.

LncRNAs in cell survival: Thousands of lncRNAs were found to be dysregulated in GC tissues compared with adjacent tissues using a microarray analysis, and several lncRNAs from the microarray were confirmed through real-time PCR assays[142,143]. Aberrantly expressed lncRNAs in GC consistently participated in key tumorigenic functions, including growth, drug resistance, and metastasis, and their presence in the tumor indicated a poor prognosis in GC patients[86,95,144,145]. The expression of several of the dysregulated lncRNAs was correlated with tumor size, TNM stage, histologic grade, differentiation, lymphatic metastasis, invasion and other classifications, including SUMO1P3[146], LINC00152[147], FER1L4[98], HMlincRNA717[97], ABHD11-AS1[148], AC138128.1[149], CCAT1[150], HIF1A-AS2[151] and more. In addition to alterations observed in GC tissue, several lncRNAs were found to have a significantly different expression pattern in serum and gastric juice. For example, CUDR, LSINCT-5 and PTENP1 were down-regulated in the serum of GC patients compared with healthy subjects[152], while AA174084 had a high expression level in gastric juice from GC patients compared with healthy controls or patients suffering from other non-cancer diseases, such as minimal gastritis, gastric ulcers and atrophic gastritis[153]. In addition, plasma H19 levels were up-regulated in GC patients compared with healthy controls but were down-regulated in postoperative specimens[154]. Together, these features reveal that lncRNAs play significant roles in the survival of cancer cells.

LncRNAs involved in cell proliferation, including HIF1A-AS2[151], MEG3[100], MALAT1[92], CCAT1[155], and LEIGC[101], were identified, however, the mechanisms by which these lncRNAs regulate cell growth are still unclear. In addition, PVT1 was up-regulated in GC tissues, and knockdown of PVT1 resulted in a significant inhibition of cell proliferation. Furthermore, PVT1 regulates the cell cycle by binding to EZH2, an important subunit of the PRC2 complex, and inhibits cyclin-dependent protein kinase inhibitors p15/p16[7]. SPRY4-IT1 was also found to be up-regulated in GC tissues and to control cell growth, colony formation, cell migration and invasion in GC cells partially through regulating the expression of cyclinD1, MMP2 and MMP9[156]. GAS5 is an lncRNA that is down-regulated in GC tissues, and ectopic expression of GAS5 in GC tumors inhibited cell growth and induced apoptosis both *in vitro* and *in vivo* through regulation of the expression of E2F1 and P21 in GC cells[99]. GHET1 physically binds to insulin-like growth factor 2 mRNA binding protein 1 (IGF2BP1), and this process promotes IGF2BP1 binding to c-Myc mRNA, resulting in increased stability of c-Myc mRNA and GC cell growth[157]. HULC, an lncRNA that is up-regulated in GC tissues and cells, is able to reduce cell apoptosis mainly by activating autophagy in the SGC-7901 cell line[158]. In addition to their role in traditional cancer growth, lncRNAs are also heavily involved in the process of drug resistance in GC. The lncRNA PVT1 is not only overexpressed in GC tissues but also in paclitaxel-resistant SGC7901cells, which indicates that it has a role in the process of GC cell resistance to paclitaxel, which so far is poorly understood[86]. In the multidrug-resistant GC cell sublines SGC7901/ADR and SGC7901/VCR, the level of MRUL was increased significantly. Knockdown of MRUL in these two multidrug-resistant sublines enhanced their sensitivity to chemotherapeutic drugs and led to an increased rate of apoptosis induced by adriamycin or vincristine[159]. AK022798 is another lncRNA involved in the resistance of GC cells to cisplatin. This lncRNA was induced by Notch 1 and overexpressed in cisplatin-resistant GC cell lines. The up-regulated AK022798 enhanced the expression of multidrug resistance- associated protein 1 (MRP1) and P-glycoprotein, thereby resulting in a suppression of apoptosis induced by cisplatin and formation of cisplatin-resistant sublines SGC7901/DDP and BGC823/DDP. This evidence suggests that AK022798 plays a significant role in the development of tumor drug resistance[10].

H19 had been demonstrated to play a critical role in GC function. H19 was found to be overexpressed in GC tissues, and it promotes GC cell proliferation[160]. Further studies suggest that c-Myc enhances the expression of H19 in GC cells, which was supported by a positive correlation between H19 and c-Myc in clinical samples[90]. MiR-675 is expressed concurrently with H19 in GC and is a known product of H19. H19/miR-675 promoted significant GC cell growth directly upon binding to RUNX1[91]. However, another mechanism of H19/miR-675 in promoting carcinogenesis has also been uncovered. Although H19 acts in a similar manner to miR-675, it was found that H19 binds to ISM1 and miR-675 also targets CALN1 in GC cells, indicating that H19 has other functions besides generating miR-675[25].

In addition to protein interactions, lncRNAs could also be interacting with miRNAs in GC cells. ANRIL is up-regulated in GC tissues and its expression is

correlated with TNM stage and tumor size in clinical samples. GC cell proliferation was significantly reduced *in vivo* and *in vitro* by reducing ANRIL expression, and the role of ANRIL in regulating cell growth was shown to be partially through inhibition of miR-99a/miR-449a levels[161]. TUSC7 suppressed the growth of GC cells by reducing miR-23b expression, which is a promoter of cell proliferation[94]. Conversely, lncRNAs can also act as targets of some miRNAs. HOTAIR is a target of miR-331-3p and miR-124 and binds with them directly in GC cells. Furthermore, HOTAIR can regulate the expression of HER2 mRNA when induced by miR-331-3p binding similar to a "sponge"[89]. Often the expression of lncRNAs is regulated by miRNAs, and for example, AC130710 is a target of miR-129-5p and is down-regulated *via* ectopic expression of miR-129-5p in GC cells[162].

Metastasis

MiRNAs as oncogenes: MiR-27 promotes GC cell metastasis by inducing EMT[163]. Moreover, single nucleotide polymorphisms (SNPs) of miRNA genes lead to functional losses or disorders of the miRNAs that are generally associated with SNPs. The G/A polymorphism in the miR-27a gene (rs11671784) directly decreases miR-27a expression. MiR-27 is responsible for directly blocking the expression of the tumor suppressor gene APC, and thus, the loss of its function contributes to EMT[164]. MiR-21 is an important oncogene that is involved in many tumorigenic factors, including metastasis and invasion, cell cycle, tumor size, and growth[165,166]. MiRNA-21 promotes tumor invasion in GC by targeting PTEN[167]. Furthermore, high levels of miRNA-21 expression are positively correlated with lymph node metastasis in GC[168]. MiRNA-21 is highly expressed in GC and is negatively correlated with PDCD4 expression, suggesting that PDCD4 is a direct target gene of miRNA-21 that inhibits cell invasion through targeted inhibition. PTEN is a well-known tumor suppressor gene that is also shown to be a direct target of miRNA-21[169]. Up-regulation of the members of miR-106b family (miR-106b, miR-93, and miR-25) in CD44(+) GC cells reduces the expression of smad7, an inhibitor of the TGF-β/Smad signaling pathway. Overexpression of miR-106b family miRNAs in CD44(+) GC cells promotes cancer stem cell-like properties and particularly EMT characteristics by activating the TGF-β/Smad signaling pathway[170]. MiR-210 is often highly overexpressed in GC and is regulated by HIF-1α. Due to this regulation, miRNA-210 expression is significantly increased in hypoxic environments where EMT develops. Unlike previously mentioned miRNAs, miR210 has been associated with *H. pylori* infection[171]. MiRNA-210 up-regulation induces significant migration and invasion of GC cells. Aside from the above-mentioned miRNAs, highly homologous miRNAs play a role in GC cell biology. For example, overexpression of miR-196a/-196b enhances GC cell migration and invasion through inhibitory oligonucleotides or direct targeting of radixin promoters in GC cells[172]. MiR-19a/b is overexpressed in GC tissues and significantly associates with the onset of metastasis. Although MXD1 is a direct target of miR-19a/b, its overexpression reduces both miR-19a/b and c-myc levels[173]. Moreover, some miRNAs directly target genes to regulate metastasis and invasion in GC cells. MiR-214 and miR-21 regulate GC cell migration and invasion by targeting PTEN[79]. In addition, miR-199a-5p acts as an oncogene in GC and functions by targeting klotho[174]. However, these are far from the only miRNA controllers that are involved in metastasis and tumor invasion through the regulation of protein signaling networks in GC cells.

MiRNAs are capable of acting as tumor suppressor genes: The miRNA-200 family suppresses GC cell metastasis by reducing the expression of the transcription factor Zeb, thereby decreasing E-cadherin expression and reducing the occurrence of EMT[175,176]. E-cadherin is a direct target of miRNA-9, and in addition, there are many miRNAs that function by targeting the EMT transcription factors Snail, Snail2, Zeb1 and Zeb2, which regulate signaling pathways controlling tumor metastasis. The tumor suppressor gene p53 can induce the expression of miR-34a and miR-192, which inhibit the expression of Snail-1 and Zeb-2, thus preventing the EMT process[177]. MiRNA-1182 targets the open reading frame of hTERT and serves to lower hTERT expression, inhibiting cell migration in GC[178]. MiRNA-146a inhibits migration and invasion in GC cells by down-regulating EGFR and IRAK gene expression[179]. In addition to these functions, miRNA-146a/b down-regulates UHRF1 by directly targeting its 3'UTR, and this effect in turn reactivates the slit homologue3, cadherin4, and RUNX3 genes *via* promoter demethylation. MiRNA-146a/b plays a key role in regulating the metastatic process in GC cells[180]. MiRNA-328-induced down-regulation of CD44v9 expression occurs in *H. pylori*-infected gastric mucosa adjacent to GC tumors, which decreases the rate at which stem cells transform into GC cells[181]. In summary, abnormal expression of miRNA has been consistently observed in GC tissues. The proteins shown to be dysregulated in GC are actually being driven by a massive miRNA expression imbalance, leading to metastasis and invasion in these tumors.

Dysregulated lncRNA expression: LncRNA HOTAIR plays a role in metastasis in GC cells. LncRNAs PCBP-1 and HOTAIR have an inverse relationship in both expression level and function. PCBP1 has been confirmed to inhibit GC pathogenesis, and overexpression of HOTAIR down-regulates PCBP1 protein levels. HOTAIR expressed in xenograft GC tumors *in vivo* increases metastasis[89,182]. In addition, HOTAIR is a known target of miR-331-3p and miR-124 and may act as a competitive inhibitor of endogenous RNAs[89]. The lncRNA H19 actively binds to ISM1,

but its expression is positively correlated with that of H19, leading to miR-675 targeting of CALN1. While overexpression of H19 directly promotes ISM1 expression and indirectly promotes miR-675 expression in GC, CALN1 is a target of miR-675. H19 mediates this process to promote GC cell metastasis[25]. In addition, lncRNAs are capable of mediating gene expression. For example, SNCG up-regulation by lncRNA AK058003 mediates hypoxia-induced GC cell metastasis. SNCG and AK058003 expression has been shown to be increased by hypoxia[183]. LncRNA HULC positively regulates GC cell migration and invasion, and the deletion of HULC reverses EMT, indicating that HULC plays a role in EMT regulation[158]. FENDRR overexpression suppresses the invasion and metastasis of GC cells by down-regulating FN1 and MMP2/MMP9 expression[96]. High linc-UBC1 expression is correlated with lymph node metastasis, and inhibition of linc-UBC1 suppresses the invasion of GC cells[184]. Silencing of SDMGC or TRIM16 decreases cell invasion and migration rates, while up-regulation of SDMGC or TRIM16 is able to promote invasion and migration[185]. Compared with the comprehensive catalogue of miRNAs uncovered, the majority of lncRNA functions are unknown and those shown to be functional have unclear mechanisms. It is understood that a few lncRNAs can mediate the expression of oncogenes or tumor suppressor genes, and dysfunction of these lncRNAs can result in GC cell metastasis and invasion. Similar to miRNAs, not only can lncRNAs take part in post-transcriptional regulatory activities by binding to the mRNA, but they can also regulate mRNAs indirectly by competitively binding with miRNAs. Currently, lncRNA research remains a small part of the overall GC field, however, we think that lncRNAs represent very important targets for clinical applications in the treatment of this disorder.

Angiogenesis

Angiogenesis is an important step during the development of cancer[186]. MiRNAs, acting as post-transcriptional regulators, are also involved in regulating angiogenesis. MiR-874, which is down-regulated in human GC, could potentially repress the expression of STAT3 by directly targeting the 3' UTR of its mRNA, resulting in a repression of the STAT3/VEGF-A pathway and significantly inhibiting tumor angiogenesis of GC cells[187]. Overexpression of miR-18a inactivated the mTOR pathway and down-regulated HIF1α and VEGF expression in SGC-7901 cells in addition to causing a substantial reduction in the number of microvessels in an SGC-7901 xenograft model[188]. MiRNA-145 also acts as an inhibitor of angiogenesis in GC cells, primarily by directly binding to 3'UTR of the Ets1 mRNA[189]. Hypoxia can induce expression of miRNAs, which may play a role in promoting angiogenesis. MiR-382, which is up-regulated by hypoxia, activates the AKT/mTOR signaling pathway by directly suppressing PTEN and therefore induces angiogenesis *via* VEGF. This evidence indicates that miR-382 is an angiogenic oncogene in GC cells under hypoxic conditions[69]. VEGF-A is a critical factor in the regulation of angiogenesis, so miRNAs that can impact VEGF-A expression should have a function in angiogenesis. For example, miR-126 was found to directly bind to the 3'UTR of VEGF-A in GC cells and therefore could inhibit angiogenesis both *in vitro* and *in vivo*[119]. In addition, lncRNAs also participate in the regulation of tumor angiogenesis. For example, MALAT1 regulates vascular growth in human endothelial cells[190], and hepatocellular carcinoma-related MVIH can activate angiogenesis *in vivo*[191]. However, thus far no lncRNAs that have been shown to impact angiogenic regulation are reported in GC cells or tissues.

CLINICAL IMPLICATIONS

MiRNAs as biomarkers

MiRNAs have the potential to be biomarkers for swift GC identification in the clinic. MiRNAs have several large advantages as biomarkers: they are highly specific, with each tissue, including tumor tissue, having its own characteristic miRNA expression profile; miRNAs are very stable and are resistant to RNAse enzymes and changes in their physical state (*i.e.*, temperature, pH and other environmental conditions); miRNAs are easy to detect, with conventional methods such as RT-PCR and gene chip analysis. However, miRNAs still have several major issues that must be addressed before they can be incorporated as cancer biomarkers: miRNA analysis cannot function on a small sample size, and requires a large-scale standardized survey; a reference range detailing the possible miRNAs active in tumors must be created; when looking for cancer metastasis, recurrence and prognosis biomarkers, it is necessary to continue to follow up with more large scale patient data to further define the cancer progression; to be used as biomarkers, detection of peripheral blood miRNAs requires the establishment of a standardized system that includes sample collection, preservation and testing to ensure the accuracy and repeatability of miRNA detection.

At present, because of the high rate of clinical GC, many researchers have opted to select patients with GC to compare their tumor tissues to their own normal tissue samples using microarray analysis. These experiments show significant miRNA increases or decreases in patients with GC. These experiments can then be used to identify key differences between cancer and normal tissue biopsy expression, and real-time PCR validation can be used to determine the accuracy, specificity and sensitivity of those markers.

MiRNAs

Potential biomarkers for GC diagnosis: To detect

differential expression of the miRNAs in GC tissues, researchers usually identify patients with GC tissues and adjacent non-transformed tissues. In addition, some researchers utilize GC tissues and compare those samples to normal tissues of other independent patients. Researchers have found that some patients' non-transformed tissue samples show increased expression of miRNAs such as miR-17, miR-106a, and miR-20a, and others show decreased expression of other miRNAs such as miR-23a, miR-150, and miR-130a[192], indicating a significant difference in miRNA profiles between non-transformed tissue samples. It is found that miR-30b[193], miR-148a[194], miR-143 and miR-195[195] are down-regulated in GC tissues compared with their matched adjacent non-tumor gastric tissues, and tend to be down-regulated in many GC cell lines. MiR-30b has the potential to be a novel tumor suppressor gene, promoting apoptosis and suppressing tumor growth by targeting plasminogen activator inhibitor-1. MiR-148a has an unknown mechanism, but could regulate several different target genes and signaling pathways involved in tumor proliferation, invasion and metastasis. MiR-375 is significantly down-regulated in distal gastric adenocarcinoma tissues and the circulating serum. In addition, this miRNA has been linked to *H. pylori* infections[196,197]. Studies have found that the levels of miR-106a and miR-21 are significantly higher in GC tissues[195,198] and are low in gastric juice[199]. Accordingly, miR-21 is likely a risk factor that could be useful for prognosis evaluation, particularly as it is also found to be similarly increased in plasma[200]. Studies have also found that many other miRNAs are differentially expressed in GC. For example, miR-31 expression is reduced, and miR-421 is overexpressed, among others. These samples have been collected from different races and regions, however, the data require more samples and appropriate statistical treatment to verify the presence of biomarkers. In addition, up-regulation of miR-21 and down-regulation of miR-133b are detectable in both gastric and esophageal cancers[201]. However, because the detection of differential expression in GC tissues is a significantly invasive procedure for patients, these markers are not suitable for initial clinical diagnosis.

Clinically, detecting differences in the serum or plasma of patients is more useful than tissue sample analysis because blood work is relatively non- invasive and easy to obtain. With more insight into blood miRNA expression during GC, it is possible to identify markers that are not only suitable for diagnosis but also can guide treatment and prognosis. In serum samples, miR-233, miR-16, and miR-100 have increased expression in patients with GC. These markers correlated significantly with clinical characteristics of GC patients, such as their TNM stages[202]. In addition, plasma miR-222 was significantly up-regulated in GC patients compared with chronic atrophic gastritis patients and healthy controls and also correlated with patients' clinical stages and lymph node metastasis status[203]. Some miRNAs that are abnormally expressed in other tumors have a similar expression pattern in GC. Circulating miR-18a is one such miRNA, which shows altered expression in both GC and bladder cancer[204]. MiR-let-7 regulation is sabotaged in GC, breast cancer[205], oropharyngeal cancer[206], and lung cancer[207]. These markers represent the potential for an extensive screening test that may be able to identify a variety of nascent cancers in patients. In plasma samples, miR-16-5p and miR-19b-3p are down-regulated, and can be used to distinguish healthy patients from GC patients with a variety of different TNM stages and differentiation grades, including the early stages of the cancer[208]. Several miRNAs found in serum samples are predictively consistent with GC standards in the industry, including mic-RNA-421, which has been shown to have a higher sensitivity and specificity than carcinoembryonic antigen and cancer antigen 125 in GC[209,210]. In addition to these markers, certain miRNAs in the blood showed altered levels unrelated to the presence of cancer, most likely due to the occurrence of hemolysis in the patient samples.

In addition to the patient's blood and stomach tissue, their gastric juice can also be used to detect increased risk of GC. Samples of gastric juice are usually collected from normal gastric mucosa, gastritis, and GC, therefore a wealth of potential biomarkers await investigation. For example, investigators found that miR-129-1-3p and miR-129-2-3p are decreased in GC[211]. In addition, miR-21 and miR-106a are decreased in GC, which also correlates with patients' TNM stage. High expression of miR-21 and miR-106a occurs in intestinal GC types compared with diffuse GC types[199]. Furthermore, miR-21 and miR-106a are also up-regulated in gastric carcinoma tissue.

Potential biomarkers of prognosis: In addition to their potential as diagnostic markers, the expression levels of specific miRNAs can also be used as prognostic markers, as several miRNA expression changes appear to suggest poor prognosis. These include the down-regulation of miR-451, which is associated with poor prognosis. Furthermore, the overexpression of miR-451 increased tumor sensitivity to radiotherapy[212]. MiRNAs can be combined with other proteins as part of a comprehensive combination marker. An example of this is miR-200c, which forms a complex with GDF15 and is indicative of poor outcomes in GC patients[213]. Many diagnostic markers may also have prognostic value; however, significant work will need to be conducted to confirm their role in prognosis.

Certain miRNAs act as key regulatory hubs, controlling a significant portion of the cancer cell's signaling network, and the reregulation of any one of them may be a marker for growth and metastasis. MiR-214 is one example of these key miRNAs[214]. Some miRNAs, such as miR-133a[123] and miR-29c[215], are known to function as tumor suppressors.

The expression of these miRNAs was significantly decreased in GC, and their deregulation was associated with lymph node metastasis in GC patients. MiR-133a suppresses TAGLN2 at the transcriptional and translational levels, while MiR-29c directly targets ITGB1. Overexpression of miR-133a inhibits cell growth and invasion and induces cell apoptosis and cycle arrest through the repression of the TAGLN2 gene, which makes it a candidate as a biomarker or a therapeutic target. Loss of miR-29c expression is an early event in the initiation of gastric carcinogenesis, which makes it attractive as a diagnostic and therapeutic biomarker for patients with GC. MiR-29c also plays a role in the efficacy of chemotherapy, and suppresses metastasis in GC[75].

Cancer stem cells are characterized by their strong tumorigenicity. In GC stem cells that express CD44, miR-106b expression is increased, activating TGF-β/Smad signaling in GC cells. This phenomenon leads to a strong invasion and migration impetus for the stem cell population. In an miRNA microarray, the miR-106b family composed of miR-106b, miR-93, and miR-25 is significantly up-regulated in CD44(+) cells compared to CD44(-) cells[170], which also significantly correlates with tumor size, borrmann type and TNM stage in GC patients[216].

LncRNAs and prognosis

In addition to miRNAs, lncRNAs can promote tumor cell expression of CD44, causing those tumor cells to exhibit stem cell properties. One example is the lncRNA GAPLINC (gastric adenocarcinoma predictive long intergenic noncoding RNA)[217]. Typically, these non-coding RNAs promote high tumor aggressiveness, and lead to a poor prognosis. GAPLINC overexpression currently defines a subgroup of GC patients with very poor survival outcomes. Mechanistic investigations show that GAPLINC regulates CD44 as a molecular decoy for miR211-3p, an miRNA that targets both molecules. Another lncRNA, ANRIL, recruits and binds to PRC2, and is up-regulated in GC tissues. Knockdown of ANRIL can repress the proliferation of GC cells both *in vitro* and *in vivo*. E2F1 induces ANRIL and ANRIL-mediated growth promotion by epigenetically silencing miR-99a/miR-449a. Like GAPLINC, the presence of ANRIL indicates a poor prognosis for patients[161]. Overexpression of the lncRNA HOTAIR is characteristic of poor prognosis in GC, and may confer an as of yet unknown malignant phenotype to tumor cells. It functions as a competing endogenous RNA to regulate HER2 expression by sponging miR-331-3p in GC[89]. HER (human epidermal growth factor receptor) based cancers, such as HER2-positive breast cancer and GC, often show miR-337 and miR-302f overexpression, and miR-139 and miR-129 underexpression[218].

In tumor cells, the regulatory interactions between lncRNAs, miRNAs and proteins are complicated and unknown. It is important not just to look at statistical markers but also to attempt to identify the mechanisms by which these molecules interact with each other to understand the full scope of the regulatory network altered by tumorigenic mutations. For now, technology is limited, but it is also important to obtain more samples for comprehensive studies.

CONCLUSION

Recently, accumulating evidence is revealing that ncRNAs play a more critical role in cancer than has been thought for decades. As we have summarized and discussed in this review, ncRNAs participate in every stage of cancer, including tumorigenesis, growth, apoptosis, cell cycle regulation, metastasis, angiogenesis, and drug resistance. Hundreds of ncRNAs have been shown to be dysregulated in tissues and cell lines, often with each sequence functioning as a tumor promoter or inhibitor. However, the mechanism driving aberrant expression has been unclear until now. We described factors that can regulate miRNA expression in GC, such as *H. pylori* infection, DNA methylation, cytokine exposure, and hypoxia. NcRNAs act as key regulators in cell processes, and their levels vary to regulate the expression levels of their targets, leading to normal growth and differentiation. Once cells suffer from carcinogenic alterations, the regulation of ncRNA levels become dysregulated, leading to cell survival in cancer lines. If the process of the cells' tumorigenesis cannot be stopped, the gene expression of these malignant cells will fundamentally change and a new ncRNA profile will emerge. This new profile often involves the down-regulation of anti-tumor ncRNAs and up-regulation of ncRNAs that promote the survival of malignant cells and the initial formation of the tumor. However, some miRNAs that are up-regulated in GC tissues and cells also act as tumor suppressors. This may be explained by the presence of a negative feedback loop, such as the case with miR-146a. It is difficult to explain the mechanism of ncRNAs in the process of malignant transformation because it is unclear whether ncRNAs are a driving force behind the transformation or a result of it. Regardless of this, the abnormal expression of ncRNAs indicates a novel method to diagnose cancer, especially in its early stages. Some ncRNAs are secreted into blood, gastric juice, or urine and this facilitates the acquisition of samples for biomarker analysis with little discomfort to the patient. Some ncRNAs can also indicate a different prognosis for patients based on their presence or their expression level in clinical testing. In addition to use as biomarkers, abnormally expressed ncRNAs may be potential candidates for cancer therapy.

Moreover, some miRNAs directly target genes to regulate metastasis and invasion in GC cells. MiR-214 and miR-21 regulate GC cell migration and invasion by targeting PTEN. MiR-199a-5p acts as an oncogene in GC and functions by targeting klotho. Some miRNAs control metastasis and invasion through the

regulation of signaling networks in GC cells. MiRNAs regulate gene expression through post-transcriptional repression and can act to control multiple cellular pathways. Based on the literature, abnormal expression of miRNA has been observed in GC tissues. The proteins that are dysregulated due to aberrant miRNA expression in GC often drive metastasis and invasion in tumor cells. The ideal time for a tumor to invade and metastasize is when the original cancer cells transform into malignancies, as that generally leads to local invasion and distant metastasis. During this process, cancer cells often undergo morphological changes and reduce their contacts to the extracellular matrix. The process by which the cancer cell undergoes metastasis and invasion is very complex. Between continuous signaling, expression of transcription factors, the reduction of cell-cell adhesion proteins such as cadherin, tumor growth, the changing tumor microenvironment, the tumor stem cells as well as indirect stimulation, it can often result in multiple mutant tumor cell genomes that compete and can accelerate metastasis and invasion of cancer cells. Of course, cancer cells grown in continuous culture do not model natural tumors without exposure to the blood vessels. These vessels function to supply oxygen to tumor cells that have limited nutritional capability, leaving hypoxic conditions within the tumor core and thus promoting tumor cell migration to the blood vessels and lymphatic areas. During metastasis and invasion, cells will detect a more suitable location to grow, although new colonies will often form at the first viable point rather than at optimal points.

Cisplatin resistance is the most common cause of treatment failure in GC patients. Many cancer chemotherapy treatments fail because of drug resistance, and miRNAs serve as modulators that can adjust the sensitivity of cells to drugs. For example, the miR-223/FBXW7 signaling pathway contributes significantly to DDP resistance of GC cells[82].

From a novel methodology in which the system is capable of identifying more screening markers and more reliable markers[219], fourteen total biomarkers have been found to be associated with GC. Additionally, three miRNAs (miR-211, let-7b, and miR-708) were reported for the first time to differentiate patients with GC and represent possible diagnostic biomarkers for GC.

Polymorphisms of miRNAs primarily occur *via* a change in their precursor molecule. Mutations of some sites can interfere the expression of mature miRNAs, which in turn impacts their function and can macroscopically affect tumor malignancy. Specific miRNA polymorphism sites can be used as tumor markers for prognosis. For example, the pri-let-7a-2 rs629367 CC genotype, which may increase the risk of GC, possibly affects mature let-7a expression and could therefore serve as a predictive biomarker for high risk and poor prognosis of GC[220]. LncRNAs are also known for their potential polymorphisms, although specific lncRNA polymorphisms are rarely studied[221]. However, research of this type is gaining more attention[222]. These biomarkers are diverse and complex and could provide the basis for future individualized treatment of GC.

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TOPIC HIGHLIGHT

2015 Advances in Liver Transplantation

Sarcopenia and liver transplant: The relevance of too little muscle mass

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Abstract

Loss of muscle mass and function is a common occurrence in both patients with decompensated cirrhosis and those undergoing liver transplantation. Sarcopenia is associated with morbidity and mortality before and after liver transplantation. The ability of skeletal muscle mass to recover after transplant is questionable, and long term adverse events associated with persistent sarcopenia have not been well studied. Limited data is available examining mechanisms by which decreased muscle mass might develop. It is not clear which interventions might reduce the prevalence of sarcopenia and associated health burdens. However, measures to either decrease portal hypertension or improve nutrition appear to have benefit. Research on sarcopenia in the liver transplant setting is hampered by differing methodology to quantify muscle mass and varied thresholds determining the presence of sarcopenia. One area highlighted in this review is the heterogeneity used when defining sarcopenia. The health consequences, clinical course and potential pathophysiologic mechanisms of sarcopenia in the setting of cirrhosis and liver transplantation are further discussed.

Key words: Sarcopenia; Liver transplantation; Cirrhosis; Body composition

Core tip: The loss of skeletal muscle mass, termed sarcopenia, is common in the setting of cirrhosis and liver transplant. Before liver transplant, it has been associated with increased morbidity and mortality. The long term effect of sarcopenia upon morbidity and mortality after transplant has been less rigorously studied. Data linking sarcopenia to adverse outcomes such as diabetes in the non-transplant setting are of interest especially with the high prevalence of post transplant metabolic syndrome. Current research on sarcopenia is limited by heterogeneity in the method to measure muscle mass and varied definitions of sarcopenia.

of too little muscle mass. *World J Gastroenterol* 2015; 21(39): 10982-10993 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i39/10982.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i39.10982

INTRODUCTION

Sarcopenia describes an involuntary loss of muscle mass and function that is observed with aging[1] and has been applied to persons with chronic diseases. In aging populations, loss of muscle mass was found to increase the risk of disability[2]. However, the consequences of sarcopenia are much greater than a decline in functional ability and include numerous adverse health manifestations. One such example is sarcopenic obesity, a condition when compared to obesity with normal muscle mass was associated with a higher risk of metabolic syndrome and coronary artery disease[3,4]. Sarcopenia is common in liver transplant candidates and recipients[5-9]. Sarcopenia in the setting of liver transplantation was associated with worse outcomes, including reduced survival[10-18]. The potential long term consequences of sarcopenia in the transplant setting, such as the development of sarcopenic obesity, are not well known. Many challenges exist in the study of sarcopenia in the setting of liver transplant and include defining sarcopenia, understanding mechanisms by which it develops and formulating preventive and therapeutic treatment options. The initial sections of this review describe the difficulty in defining sarcopenia and the heterogeneity in clinical studies relating to liver transplantation. Later sections in this review focus on the health consequences of sarcopenia in the transplant setting and potential mechanisms by which sarcopenia might develop.

HETEROGENEITY IN DEFINING SARCOPENIA IN THE SETTING OF CIRRHOSIS AND TRANSPLANT

The concept of sarcopenia is straightforward, representing a threshold of diminished lean body mass. Although the term sarcopenia is widely used, methods to assess sarcopenia and the thresholds to define it differ significantly. A common definition of sarcopenia is measured muscle mass two standard deviations below a reference range obtained from a young and healthy population[2]. However, measuring muscle mass alone does not account for the loss of muscle function that occurs with sarcopenia. Recognizing this limitation, the Society of Sarcopenia, Cachexia and Wasting Disorders in 2011 added a definition of "sarcopenia with limited mobility". Sarcopenia with limited mobility was defined as lean appendicular skeletal mass corrected for height greater than 2 standard deviations below the mean of healthy persons aged 20 to 30 years of the same ethnic group and walking speed less than 1 meter per second or total distance walked during a six minute walk test less than 400 m[19]. Investigators examining muscle loss in the setting of cirrhosis and liver transplantation have used both quantitative and functional measures of muscle.

Further complicating research on sarcopenia in the setting of cirrhosis and liver transplantation are different methods to quantify muscle mass and muscle function. Some measures of muscle mass only account for a single anatomic area, such as single slice imaging or limb anthropometry. Other measures, including dual X-ray absorptiometry (DXA) and bioelectrical impedance analysis (BIA) are based on total body measurements. Often, total body measures are limited by their ability to measure different body compartments. Understanding the composition of these compartments is important as methods often measure skeletal muscle as a component of one compartment. For instance, some tests are only able to distinguish two compartments, fat mass and fat free mass. In a two compartment model, fat free mass consists of lean tissue, body water and bone mineral content. Many measures of body composition make assumptions on the proportion of total body water within lean body mass. In cirrhosis, when volume overload is present, the proportion of total body water is altered resulting in these methods being less accurate[20]. A combination of methods measuring four compartments is the most accurate means to measure body composition in cirrhosis with altered hydration[8,20]. These four compartments include fat mass and three compartments of fat free mass consisting of total body water (which can be divided into intracellular and extracellular water), total body protein and bone mineral content.

IS THERE A "GOLD STANDARD" MEASURE OF BODY COMPOSITION IN CIRRHOSIS?

Overall, there is no data to support any singular measure of body composition as a "gold standard" in the setting of cirrhosis and liver transplantation. As noted above, a four compartment model is likely required to make precise individual measurements of body composition in the setting of cirrhosis. This typically entails using combinations of measures, including a direct measure of total body water. Total body water can be directly measured by techniques such as radiolabeled water[8,21]. Measuring total body water is important to account for changes in total body water that occur with worsening severity of liver disease[8]. Early in the course of liver disease, excess fluid is mainly extracellular water[22], which was found

Table 1 Methods used to define muscle mass or function in cirrhosis

Method	Measurements obtained	Limitations	Notations
Multiple (four) compartment model	Total body water Body fat Fat free dry matter (protein) Bone mineral content	Requires combinations of methods (such as water dilution, densitometry)	Best model for cirrhosis when fluid overload is present
Dual X-ray Absorptiometry	Body fat Fat free body mass Bone mineral content	Limited ability to differentiate between lean tissue and body water with excess body water resulting in overestimation of fat free mass[1] Ascites, especially more than 4 liters, can significantly impact truncal measures[26,27]	Peripheral measures of lean tissue are less impacted by ascites[24,26]
Cross sectional imaging	Estimate skeletal muscle volume	Can be used to determine differences in skeletal muscle between groups[31] Studies use different muscle groups, anatomic levels and cutoff values to diagnose sarcopenia	Measurements 5 cm above the level of the 4th-5th lumbar vertebra had the highest correlation with total body skeletal volume[31]
Biochemical methods	Skeletal muscle mass	Total body protein is a measure of functional muscle mass and can be done through techniques such as *in vivo* neutron activation analysis 24 h urine creatinine is one method, but is limited in cirrhosis where renal insufficiency is common[93]	Use calculations based on these methods to quantify muscle mass
Bioelectrical impedence analysis	Fat free mass Fat mass	Guidelines recommend against routine use of BIA under states of altered hydration[94]	Segmental BIA was found to have a better correlation and lower standard error in estimating body cell mass in the setting of cirrhosis without ascites but still performs poorly with ascites present[95]
		BIA estimates of total body water were found to be accurate in cirrhotic patients without ascites, but performed poorly when ascites was present[23]	Phase angle can be used. In a study including participants with a wide range in severity of liver disease, phase angle was positively correlated with total body protein, muscle mass and muscle strength[96]
Anthropometry	Estimate muscle mass	Edema alters results of anthropometry overestimating muscle mass[8]	Mid arm circumference was found to be one of the most accurate anthropometric measures[29] and was most predictive of clinical outcomes[97]
Functional Measures	Measures ability to perform physical task, for which muscle function is one component	Common functional measures assess all systems involved in exercise including cardiovascular, pulmonary, hematologic, neurologic and musculoskeletal[33]	Include tests such as submaximal cardiopulmonary fitness tests, six minute walk test, hand grip strength and isokinetic strength of flexion and extension at different joints Often simple tests such as hand grip correlate with measures of skeletal muscle[29]

BIA: Bioelectrical impedance analysis.

to increase in patients with cirrhosis even without clinically apparent edema or ascites[23]. Any increase in total body water can lead to the overestimation of lean body mass.

Often singular measures are chosen to estimate skeletal muscle. This is especially relevant when serial measures are being performed in a cohort. Limitations to using a singular test must be understood. Those limitations exist in both the methods of quantifying muscle and the definitions of sarcopenia. Table 1 highlights methods and limitations in techniques measuring muscle mass in cirrhosis. DXA and cross sectional imaging appear to have the most utility when using a single technique. In the non-transplant setting, a commonly accepted method to quantify sarcopenia is DXA[2]. Many investigators studying liver transplantation used cross sectional imaging, with the majority of data derived from this method. These two methods are discussed in further detail.

DXA is considered a gold standard examination of body composition in the non-transplant setting[2,24,25], however, it is recognized that fluid overload and ascites can impact the reliability of this technique. DXA can measure body fat, fat free mass and bone mineral content. Limitations in the ability to differentiate between lean tissue and body water are the main drawback to this technique[1] as excess body water may result in the overestimation of fat free mass. The greatest effect of volume overload and ascites upon measurements occurred in those made from the trunk[26-28]. This is particularly true when the ascites volume was greater than four liters[26]. Peripheral measures obtained by DXA, which is how sarcopenia is often quantified, were influenced less by ascites. Additionally, measurement of peripheral lean tissue mass was not altered after draining ascites[24,26]. In groups with cirrhosis, DXA was found to be more than 80% accurate in determining depleted body cell

mass[29]. When comparing DXA with anthropometry, single-frequency BIA, multiple frequency BIA and whole body gamma counting against gold standard multi-compartment measures of fat free mass in a cohort of patients with cirrhosis, DXA was found to have the smallest bias[30].

Cross sectional imaging has been frequently applied when studying sarcopenia in the setting of cirrhosis and liver transplantation. This method is appealing as imaging studies are often done for clinical indications during the care of patients with cirrhosis or around the time of liver transplantation. Single slice imaging was found to correlate highly with whole body skeletal muscle (r = 0.924)[31]. However, deviation in measurements obtained from single slice imaging compared to whole body imaging make this technique better for group comparison, as opposed to individual characterization, of muscle mass[31]. This method is additionally limited by the lack of a standardization in the technique of measuring muscle volume and the heterogeneity in defining sarcopenia (shown in Table 2). Muscle measurements 5 cm above the level of the 4th-5th lumbar vertebra were found to have the highest correlation with total body skeletal mass[31]. Imaging is often done based on clinical indications, and serial computed tomography measurements may be influenced by selection bias. A notable advantage of this method is the ability to measure quantity and quality of muscle simultaneously[14]. This can be done through measuring intramuscular adipose tissue content[32].

Functional measures of muscle are another common assessment undertaken in cohorts with cirrhosis. Measures of muscle function often measure strength of localized muscle groups or overall performance. One advantage is many of these measures such as six minute walk testing (SMWT) or hand grip dynamometry can be done quickly with little expense in large numbers of patients. The main disadvantage is many of these tests measure all systems involved in exercise or movement including cardiovascular, pulmonary, hematologic, neurologic and musculoskeletal[33]. For example, decreased performance on a submaximal exercise test could result from hepatopulmonary syndrome, cirrhotic cardiomyopathy, and sarcopenia simultaneously present in an individual transplant candidate.

CLINICAL CONSEQUENCES OF SARCOPENIA IN THE SETTING OF CIRRHOSIS AND TRANSPLANT

Measured decreases in both muscle mass and muscle function have been associated with multiple adverse clinical outcomes as shown in Table 2 and Table 3. Table 2 highlights the varied definitions of sarcopenia used in cirrhosis and transplant. Both tables highlight key outcomes and limitations in the study of sarcopenia in cirrhosis and transplant. Studies of muscle mass[5-9] and function[34-37] were consistently found to be decreased in persons with cirrhosis and post liver transplant when compared to healthy control populations. When assessed by gender, decreases in lean body mass may be more pronounced in men[7]. Decreased measured skeletal muscle mass[10-18], muscle function[34,36,38] and performance during cardiopulmonary testing[39] all have been associated with pre- and post-transplant mortality. Decreased muscle quality, defined as increased intramuscular adipose tissue, was additionally associated with higher post transplant mortality[14]. The effect of diminished muscle mass and function on survival appears to be mostly independent of model for end stage liver disease (MELD) or Child-Pugh classification[12,40]. However, as the severity of liver disease increased, the effect of sarcopenia on survival might diminish. For example, one study found sarcopenia was associated with mortality only when MELD was less than 15[12]. Measures of muscle function also capture the risk of mortality in patients with debility, to which sarcopenia contributes. Distance ambulated on a six minute walk test less than 250 m was independently associated with increased mortality[34,36], and it was found that wait list survival improved for every incremental increase of 100 m walked (HR = 0.58, 95%CI: 0.37-0.93)[34]. Nutrition may be one means to improve these observed outcomes with sarcopenia. In patients with pretransplant sarcopenia, nutritional supplementation, including branched chain amino acids, provided before and after transplant was found to improve post transplant survival[11].

The loss of muscle mass and muscle function tends to occur as patients with cirrhosis develop complications typical of end stage liver disease. Although associations do not equate causality, many measures of decreased muscle mass or function were linked to complications of cirrhosis. Decreased hand grip strength alone was found to be associated with complications including ascites, hepatorenal syndrome and spontaneous bacterial peritonitis[41]. Another study found an association between anthropometric measures of malnutrition with psychomotor assessments of hepatic encephalopathy[42]. However, another series found no association between body composition characterized by anthropometry and BIA with hepatic encephalopathy[43]. One would predict as the severity of liver disease increases, the risk of sarcopenia would also increase, however, the relationship is not straightforward. Muscle function was found to be inversely correlated with Child-Pugh classification[36] or MELD score in some studies[34]. However, this observation is not universal. Other studies found a minimal or complete absence of an association between MELD or Child-Pugh classification and muscle mass or function[11,39]. In another series, sarcopenia increased with Child-Pugh classification, but was not correlated with MELD[12]. This difference may be

Table 2 Measures, definitions and outcomes relating to sarcopenia in the setting of cirrhosis and liver transplant

Study	Method	Definitions used/proposed	Outcomes	Notes/Limitations
Selberg *et al*[96]	BIA, phase angle	> 5.4° normal 4.4°-5.4 borderline < 4.4° abnormal	Phase angle < 5.4° associated with significantly lower survival	Phase angle may remain normal in cases of severe tissue loss when proportional losses of extracellular mass and body cell mass may occur
Kaido *et al*[11]	BIA, multiphase device (InBody 720; BioSpace, Tokyo, Japan) Percent skeletal muscle mass against a standard and calculated body cell mass	< 90% skeletal muscle mass compared to standard or body cell mass below 23.0 kg	Survival was significantly decreased in recipients with low skeletal muscle mass or low body cell mass	No data is provided on volume status, although Child-Pugh classification is given Nutritional supplementation with branched chain amino acids improved survival in those with low skeletal muscle mass
Englesbe *et al*[15]	CT, combined area of right and left psoas muscle area at the highest level of the 4th lumbar vertebra Control population was 248 trauma patients	Percentile cutoffs for total psoas area in transplant population 1910 mm^2 50th percentile 1420 mm^2 25th percentile 950 mm^2 5th percentile	Decreased psoas muscle area associated with higher risk of mortality 25th percentile HR = 1.88 5th percentile HR = 3.46	Retrospective definitions of sarcopenia were not derived from the control trauma patients, but were based on percentiles from the transplant population Included CT scans either 90 d before or after transplant; majority of scans were after transplant
Tandon *et al*[12]	CT or MRI, cross sectional area of muscle at 3rd lumbar vertebra (psoas, paraspinals, transversus abdominis, rectus abdominis and internal and external obliques)	Total L3 skeletal muscle area $\leqslant$ 52.4 cm^2/m^2 in males $\leqslant$ 38.5 cm^2/m^2 in females	Sarcopenia present in 41% of wait listed candidates Higher wait-list mortality with sarcopenia (HR = 2.36, 95%CI: 1.23-4.53) Greatest effect was in those with low MELD score	Retrospective Only study to report use of both MRI and CT
Montano-Loza *et al*[18]	CT cross sectional area of muscle at 3rd lumbar vertebra (psoas, paraspinals, transversus abdominis, rectus abdominis and internal and external obliques) Muscle identified by Housfield unit between -29 and + 150	Total L3 skeletal muscle area $\leqslant$ 52.4 cm^2/m^2 in males $\leqslant$ 38.5 cm^2/m^2 in females	Sarcopenia present in 40% of cirrhotics Sarcopenia was independent risk factor for mortality (HR = 2.28, P = 0.008) One year survival for cirrhosis with sarcopenia was 53% compared to 83% in cirrhosis without sarcopenia	Prospective data
Hamaguchi *et al*[14]	CT, cross sectional psoas muscle area at level of umbilicus Intramuscular fat accumulation of multifidus muscle (multifidus muscle Housfield units/ subcutaneous fat Housfield units)	ROC curves selected from study data for best accuracy in predicting death Intramuscular adipose tissue content -0.375 in males and -0.216 in females Psoas muscle mass normalized for height $\leqslant$ 6.868 cm^2/m^2 in males $\leqslant$ 4.117 cm^2/m^2 in females	Pretransplant increased intramuscular adipose tissue content (OR = 3.898, 95%CI: 2.025-7.757) and decreased psoas muscle mass (OR = 3.635, 95%CI: 1.896-7.174) were associated with mortality	Used umbilical level which can vary based on body habitus Constructed cutoffs based on diseased population Included intramuscular fat content as a measure of muscle quality
Tsien *et al*[13]	CT cross sectional at mid 4th vertebra level	Psoas muscle area normalized 5th percentile cutoffs $\leqslant$ 12.27 cm^2/m^2 in males less than 50 yr of age	Sarcopenia was seen in 62.3% prior to transplant and increased to 86.8% after transplant	Includes serial measures in the same patients
	Total cross sectional area of psoas, paraspinals and abdominal wall muscles (rectus abdominis, oblique and transversus abdominis) normalized to height	$\leqslant$ 10.12 cm^2/m^2 in males more than 50 yr of age $\leqslant$ 10.47 cm^2/m^2 in females less than 50 yr of age $\leqslant$ 10.33 cm^2/m^2 in females more than 50 yr of age	Only 6.1% had reversal of sarcopenia after transplant and 75% without pretransplant sarcopenia developed it after transplant	Mean time from transplant to post-transplant CT was about one year (13.1 ± 8.0 mo)

	Reference ranges derived from 109 healthy control subjects undergoing CT for unspecified abdominal pain	Total abdominal muscle area normalized 5th percentile cutoffs $\leqslant$ 60.09 cm^2/m^2 in males less than 50 yr of age $\leqslant$ 48.97 cm^2/m^2 in males more than 50 yr of age $\leqslant$ 53.43 cm^2/m^2 in females less than 50 yr of age $\leqslant$ 41.28 cm^2/m^2 in females more than 50 yr of age	Reduction in muscle after transplant was associated with new onset diabetes mellitus	Since follow up scan was done for indications (ie HCC surveillance, infection, pain, increased aminotransferases) the potential for significant selection bias exists
Masuda *et al*[9]	Cross sectional CT of psoas muscle at L3 Calculated area by multiplying major and minor axis of psoas (a × b × ∏) Compared to a reference group of healthy donors	< 800 cm in men < 380 cm in women	3 and 5 yr survival with sarcopenia was 74.5% and 69.7% respectively, without sarcopenia was 88.9% and 85.4% respectively (P = 0.02) Sepsis was seen in 17.7% with sarcopena, 7.4% without sarcopenia (P = 0.03)	Enteral nutrition given in immediate post operative period appeared to decrease risk of sepsis when sarcopenia was present

MELD: Model for end stage liver disease; CT: Computed tomography.

explained by the inclusion of the degree of ascites in the Child-Pugh classification, a factor which has a large effect on nutrition.

The presence of sarcopenia predicts more complicated hospital stays during the transplant period. Diminished performance observed during cardiopulmonary testing was associated with longer intensive care unit stays[39]. Decreased hand grip strength and diminished lean body mass noted on a DXA study were also associated with longer intensive care unit stays[44]. Multiple factors related to sarcopenia may account for prolonged intensive unit care stays, and includes respiratory muscle weakness which was observed in patients with cirrhosis and high MELD scores[45]. A higher rate of sepsis was observed in the setting of sarcopenia in one series[9], a complication that could account for both morbidity and mortality. Nutritional interventions represent a potential means to mitigate the effects of sarcopenia upon sepsis. Early after receiving a live donor liver transplant supplemental enteral feeding reduced the risk of sepsis in the group with sarcopenia from 28.2% to 10.5% (P = 0.03) in one study[9].

The consequences of sarcopenia extend beyond survival and the hospital stay. Skeletal muscle plays a critical role in insulin sensitivity. In the non-transplant setting, population based data showed sarcopenia was associated with insulin resistance and metabolic syndrome[4,46]. The prevalence of the post-transplant metabolic syndrome ranges from 44%-58%[47-51], and it is possible that sarcopenia may be a significant contributing factor. As an example, reduced psoas muscle area after transplant was associated with a 3 fold higher risk of developing diabetes mellitus[13]. The effect of sarcopenia on the health of long term survivors after liver transplant has not been well studied.

CLINICAL COURSE OF SARCOPENIA AFTER TRANSPLANT

Increased weight and body mass index are commonly observed after liver transplantation[35] with one series finding the median weight gain at 1 and 3 years was 5.1 kg and 9.5 kg, respectively[52]. Most weight gain occurs within the first year after liver transplant[53-55], and it appears that much of this weight gain is an increase in fat mass. Longitudinal data differs on changes in lean body mass after liver transplant with some series showing continued decreases in lean body mass[13,56-58], some studies finding increased lean body mass[59,60] and one without significant change[61]. Decreased lean mass does not always reach the threshold of sarcopenia, and one study examined the rate of sarcopenia before and after transplant. In that study, the prevalence of sarcopenia, defined by cross sectional imaging, increased from 62.3% pretransplant to 86.8% post-transplant roughly one year after transplant[13]. Most longitudinal studies have examined outcomes within the first one to two years after transplant. Less information is known about changes long term in body composition. In a group of long term survivors after liver transplantation, measurements of body composition in many transplant recipients were similar to those seen with sarcopenic obesity[62]. In another study of long term survivors (> 10 years) after transplant, body mass index identified 7.3% as malnourished whereas bioimpedance identified 31.7% as malnourished, highlighting how a gain in fat mass may result in the under recognition of persistent sarcopenia[63].

Muscle function improves after transplant with increases in maximal oxygen uptake[35,59,60], distance walked during six minute walk testing[35] and isokinetic joint strength being observed[35,59]. Most of these

Table 3 Methods and outcomes when measuring muscle function in the setting of cirrhosis and liver transplant

Study	Method	Outcomes	Notes/limitations
Andersen *et al*[37]	Isokinetic strength of flexion and extension of six joints	Upper and lower extremity strength was decreased in cirrhotics *vs* controls	Only included patients with alcohol related cirrhosis
		Lower extremity strength was associated with lean body mass and mid arm circumference, an effect independent of severity of liver disease, neuropathy, biochemical data and recent alcohol use	The majority of patients had Child-Pugh A or B classification Included 24 cirrhotics and 24 controls
Tarter *et al*[98]	Isokinetic strength measured by upper and lower extremity peak force, peak torque, total work and power	Most measures of strength were decreased in cirrhotic patients *vs* controls	Study included 49 with alcoholic cirrhosis, 42 with non-acoholic cirrhosis and 50 controls
		There was no difference in any measure between those with alcohol *vs* non-alcohol related cirrhosis	No patient had consumed alcohol in greater than one year prior to testing
Beyer *et al*[35]	Maximal oxygen uptake measured on a cycle ergometer SMWT Isokinetic knee flexion and extension	Maximal oxgen uptake, SMWT and isokinetic knee strength increased over the first six months after transplant compared to pretransplant values	Small study with only 17 patients having post transplant data and 13 patients completing both pretransplant and posttransplant assessment of maximal oxygen uptake
		No changes were noted between six and 12 mo after transplant	Used a supervised exercise program after transplant
Epstein *et al*[38]	Symptom limited cardiopulmonary testing on a cycle ergometer	When examining patients that went on to transplant, a significantly higher proportion of patients that died within the first 100 post-operative days had a peak oxygen consumption < 60% predicted and had oxygen consumption at the anaerobic threshold < 50% predicted peak oxygen consumption	Median MELD at the time of exercise testing was low (7-12) The median time from exercise testing to transplant was long (471 ± 300 d)
Prentis *et al*[39]	Symptom or exertional limited cardiopulmonary testing on a cycle ergometer	Sixty tested patients went on to liver transplant with a 10% 90 d mortality	Mean MELD at transplant was low (< 20)
		Mean aerobic threshold was higher in survivors and was only variable in multivariate analysis that was associated with mortality	Compared to above study (Epstein 2004[38]), the authors did not make comparisons to population based reference values, but used ROC curve analysis to define thresholds associated with outcomes
		Optimal anaerobic threshold associated with survival was > 9 mL/min per kg Anaerboic threshold > 11 mL/min per kg was associated with shorter stay in critical care setting	
Carey *et al*[34]	Six minute walk test	Candidates awaiting liver transplant had decreased SMWT distance (369 ± 122 m), significantly lower than reference values	Included patients too ill to walk, and designated zero m for this group
		When controlling for other factors including age and MELD, SMWT distance was significantly associated with wait list mortality (HR = 0.58, 95%CI: 0.37-0.93)	
		ROC analysis found cut off value of 250 m having the highest sensitivity and specificity for mortality	Designated patients removed from the list as a waitlist death
Alameri *et al*[36]	Six minute walk test	Patients with cirrhosis had significantly diminished SMWT distance (306 ± 111 *vs* 421 ± 47 m, $P < 0.0001$)	Used Child-Pugh to assess severity of liver disease, no data on MELD
		SMWT was an independent predictor of survival and was inversely correlated with Child-Pugh classification	
		The lowest quartile walked < 250 m	

SMWT: Six minute walk test; MELD: Model for end stage liver disease.

improvements occur early, within the first six months after liver transplant[35]. In one series comparing SMWT at 7 and 14 d after transplant, mean SMWT increased nearly 100 m[64]. Such rapid improvement in functional testing would suggest much of this early recovery is independent of increases in muscle mass. In studies measuring skeletal muscle mass and hand grip strength, hand grip values rapidly improved within the first 3 mo after transplant whereas skeletal muscle remained below pretransplant values[58].

POTENTIAL MECHANISMS BY WHICH SARCOPENIA MIGHT DEVELOP AND PERSIST IN THE SETTING OF LIVER CIRRHOSIS AND TRANSPLANTATION

There are numerous mechanisms by which sarcopenia might develop in patients with cirrhosis. Findings of both increased muscle protein catabolism[65,66] and decreased muscle protein synthesis[67] were observed in cohorts with cirrhosis. Malnutrition is commonly observed in patients with cirrhosis. Dietary total caloric intake is often decreased with consumption of protein, carbohydrate and fat observed to be less than healthy controls[68]. Coupled with decreased intake, increased protein requirements and increased amino acid catabolism often observed with cirrhosis suggest inadequate stores of hepatic glycogen which result in gluconeogenesis from amino acids[6,69]. Further evidence of reduced glycogen stores comes from observing the rapid development of a catabolic state after short periods of fasting in cirrhotic patients[70,71]. Poor absorption of nutrients could contribute to malnutrition. For example, malabsorption of fat was found to occur in patients with cirrhosis even with normal intestinal mucosal function[72]. Benefits of nutritional supplementation in patients with cirrhosis highlight the role of malnutrition in the development of sarcopenia. Night time snacking was found to decrease skeletal muscle proteolysis, but study results differed when improvements in skeletal muscle mass were examined[73].

Branched chain amino acids are another means of supplementation. Activation of the mammalian target of rapamycin (mTOR)/Akt pathway was found to result in muscle hypertrophy and regeneration[74,75]. Increased availability of branched chain amino acids, particularly leucine, were found to stimulate Akt/mTOR[76,77]. In a randomized trial, supplementation of branched chain amino acids in patients with cirrhosis was associated with increased triceps skin fold thickness and decreased mean Child-Pugh scores, an effect not seen with carbohydrate or other protein supplementation[78]. It is not clear if a particular deficiency in branched chain amino acids exists in patients with cirrhosis outside of the overall state of malnourishment observed.

Portal hypertension was shown to have a significant role in malnutrition seen with cirrhosis. In animal models, portal hypertension was found to decrease intestinal absorption of carbohydrates[79]. Ascites is another manifestation of portal hypertension that contributes to malnutrition and muscle loss. Measures causing resolution of ascites, such as the placement of a transjugular intrahepatic portosystemic shunt, were found to improve body nitrogen measures of malnutrition, but interestingly did not result in increased muscle strength[80]. Nutritional supplementation can be another means to combat the effect of ascites upon muscle mass. Patients with cirrhosis and refractory ascites were found to have preserved anthropometric measures of lean body mass along with improved survival when provided with parenteral nutrition for 24 h after large volume paracentesis, an effect not seen with enteral nutritional support[81].

The etiology of liver disease may also play a role. Alcohol abuse can induce both a neuropathy[5] and a myopathy[5,82]. However, the pathophysiology of these changes in patients with alcohol induced liver disease is complex. This is illustrated by a study of men with alcoholism where muscle strength was related to total lean body mass, but not to the presence of neuropathy or the degree of liver disease[37]. Viral hepatitis may also impact muscle function as non-cirrhotic patients with either chronic hepatitis B or chronic hepatitis C found to have decreased SMWT distance compared to healthy controls[36]. Reasons for this decreased performance on the SMWT in those with chronic viral hepatitis were not examined.

After liver transplantation, immunosuppression contributes to both ongoing muscle loss and delayed regeneration. Calcinuerin inhibitors (CNIs), the mainstay of liver transplant immunosuppression regimens, have multiple effects on muscle. Intracellular calcinuerin activation regulates genes involved in skeletal muscle maintenance, growth and remodeling[83]. Administration of calcinuerin inhibitors was found to inhibit skeletal muscle hypertrophy in animal models after the administration of growth factors or during periods of increased muscle work[84,85]. CNIs additionally were found to prevent a switch in the type of muscle fiber from slow to fast fibers[86]. Myostatin appears to be one important mediator between CNI use and sarcopenia. Myostatin inhibits muscle growth and regeneration[87], an effect mediated through reduced satellite cell activation[88]. CNI use, in animal studies, was found to increase myostatin expression, a growth differentiation factor which inhibits muscle growth[89]. Small pilot data in transplant recipients found increased skeletal muscle myostatin expression compared with controls[13].

Although CNIs are the most commonly used immunosuppressive agents after transplant, other agents affect muscle. Inhibitors of mTOR, such as rapamycin, were found to block muscle hypertrophy[75].

Steroids have long been described to result in myopathy which is histologically characterized by type Ⅱ muscle fiber atrophy[90]. Although not commonly reported, the anti-metabolite mycophenolate mofetil was implicated in a case report of a medication induced myopathy that reversed with the withdrawal of this agent[91].

Lifestyle may also play a significant role in the persistence of sarcopenia after transplantation. Many transplant recipients remain sedentary[51]. Even when enrolled in a study to assess the benefit of exercise after transplant, less than half adhered[60]. However, among those that did adhere with exercise recommendations, there was a trend toward improved body composition[60]. The best type of exercise has not been defined after transplant. Much focus has centered on aerobic fitness. In the non-transplant setting, resistance exercise has been noted to increase muscle protein synthesis[92]. Exercise before and after transplant could offer a means of both prevention and treatment.

CONCLUSION

The development of sarcopenia is common in patients with cirrhosis. It is associated with numerous adverse events including wait list and post-transplant mortality. A key area in the study of sarcopenia and cirrhosis is standardization of methods and definitions of sarcopenia in this setting. Further investigations should focus on the pathophysiologic basis in which sarcopenia develops and persists in the setting of liver disease and liver transplant. Special focus should be maintained on modifiable risk factors which could include diet and physical activity interventions. Finally, more data is needed on the long term effects of sarcopenia after transplant, especially in light of the high rate of metabolic syndrome and cardiovascular events observed in this population.

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TOPIC HIGHLIGHT

2015 Advances in Liver Transplantation

Liver transplantation for alcoholic liver disease: Lessons learned and unresolved issues

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Abstract

The use of liver transplantation (LT) as a treatment for alcoholic liver disease (ALD) has been highly controversial since the beginning. The ever increasing shortage of organs has accentuated the low priority given to patients suffering from ALD, which is considered a "self-inflicted" condition. However, by improving the long-term survival rates, making them similar to those from other indications, and recognizing that alcoholism is a primary disease, ALD has become one of the most common indications for LT in Europe and North America, a situation thought unfathomable thirty years ago. Unfortunately, there are still many issues with the use of this procedure for ALD. There are significant relapse rates, and the consequences of excessive drinking after LT range from asymptomatic biochemical and histological abnormalities to graft failure and death. A minimum three-month period of sobriety is required for an improvement in liver function, thus making LT unnecessary, and to demonstrate the patient's commitment to the project, even though a longer abstinence period does not guarantee lower relapse rates after LT. Recent data have shown that LT is also effective for severe alcoholic hepatitis when the patient is unresponsive to corticosteroids therapy, with low relapse rates in highly selected patients, although these results must be confirmed before LT becomes a standard procedure in this setting. Finally, LT for ALD is accompanied by an increased risk of *de novo* solid organ cancer, skin cancer, and lymphoproliferative disorders, which has a large impact on the survival rates.

Key words: Liver transplantation; Alcoholic liver disease; Cirrhosis; Alcoholic hepatitis; Survival rates; Relapse; Six-month rule; Sobriety; Solid organ cancer

Core tip: Alcoholic liver disease (ALD) has become one of the leading indications for liver transplantation (LT) over the last twenty years. In the context of scarcity of organs, the excellent survival and compliance rates of LT for ALD make this a favorable procedure. However, there are considerable relapse rates, which can have dire consequences, such as graft loss and death. Other issues have also emerged: increased risk of malignancy, concomitant hepatitis C virus infection, and LT for alcoholic hepatitis. This review will first discuss the highly controversial history of LT for ALD and then focus on the main questions that remain unanswered in 2015.

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INTRODUCTION

Alcohol consumption accounts for 3.8% of global mortality and 4.6% of disability-adjusted life-years (DALYs) lost due to premature death[1]. Among the various harmful effects of alcohol, alcoholic liver disease (ALD) induces a wide spectrum of liver abnormalities, including simple steatosis, alcoholic hepatitis or steatohepatitis, progressive fibrosis, and ultimately cirrhosis and/or hepatocellular cancer (HCC)[2]. At least 30% of all steatosis cases can be attributed to alcohol, according to a population-based French study[3]. The risk of developing ALD increases in a dose-dependent manner, is higher among women, and generally appears after several years of consumption of > 7-13 beverages per week for women and 14-27 beverages per week for men, according to a Danish prospective study including 13285 individuals over a 12-year follow-up period[4].

The current management of alcoholic cirrhosis comprises abstinence, nutritional therapy rich in calories and proteins[5], and prophylaxis of any associated complications. Despite this management strategy, hepatic decompensation, disease progression, and life-threatening complications (variceal bleeding, bacterial or fungal infections, hepatic encephalopathy, and HCC) can occur, and liver transplantation (LT) may be needed. ALD, alone or in combination with other liver-related diseases such as hepatitis B or C viruses and NASH, is one of the most common indications for LT in Europe (23% of all LT between 1999-2009 were at least partially attributable to alcohol abuse, according to the European Liver Transplant Registry ELTR[6]) and North America (24.1% of all procedures in 2013[7]). Once decried[8], LT for ALD has now become an accepted, safe, and common procedure, with excellent survival and reasonable relapse rates[9]. It is at least partially responsible for the decrease seen in liver-related mortality[10,11].

BRIEF HISTORY AND CONTROVERSY

Before the advent of cyclosporine, the first report of survival rates from post-LT ALD patients indicated that the short-term prognosis was poor compared to other indications. Of the first ten patients who received a transplant by Starzl *et al*[12], nine did not survive the first four months. This was probably due to the selection of critically ill patients who were too sick for improvement even with the intervention. In 1983, the National Institutes of Health predicted that ALD would be a marginal indication for LT. In 1984, Scharschmidt[13] reported the experience of four transplant centers that had performed 540 transplantations in the United States and Western Europe. The three-year survival rate for the 20 transplant patients after 1980 was 20%; non-alcoholic cirrhosis had an impressive 42% survival rate. The first positive data published about the survival rate of ALD patients after LT, in comparison to other indications, came in 1988. Starzl *et al*[14] reported a 73% one-year survival rate among 41 patients when cyclosporine was used as the main immunosuppressive drug. This was confirmed by numerous European and American centers in the early nineties, with one-year survival rates from 66%-96%[15-22].

Since the beginning, the use of LT to treat alcoholic cirrhosis has been highly controversial. The naysayers believed that alcohol consumption had concomitant multisystem organ consequences that precluded a good result from surgery, relapse induced redevelopment of the liver disease, and patients were unlikely to withstand the psychological issues caused by such a serious operation, resulting in poor compliance[23,24]. Further taking into account the shortages of available grafts and high cost of LT, many specialists considered it unacceptable to "waste" grafts on alcoholics who were responsible for the harm caused to their liver. This began to change as alcoholism became accepted as a primary chronic disease by the medical community[25], whereas hitherto it was considered a vice[26].

TIMING OF REFERRAL FOR TRANSPLANTATION

One of the main issues surrounding LT for ALD is identifying the ideal time to consider the operation. The first parameter to consider is the severity of the disease. The benefit of LT for alcoholic cirrhosis is limited to patients with advanced decompensation

(*i.e.*, a Child-Pugh score of 11-15[27] or MELD score > 11[28]). A randomized controlled trial conducted at 13 living donor liver transplantation centers in France that included 120 Child-Pugh stage B patients showed that immediate listing for LT did not improve the five-year survival rates, and it increased the risk of extrahepatic cancer compared to standard care[29].

There are few therapeutic options for ALD, and the main treatment is complete abstinence[30]. Therefore, improvement can only be expected in about 66% of Child-Pugh C patients after the first episode of decompensation. This improvement concerns hepatocellular functions and portal hypertension, and these improvements should be visible in the first three months following the discontinuation of alcohol use[31].

Whether or not the patient remains in end-stage liver disease or has other life-threatening complications, LT should be encouraged as soon as possible. This is the natural course in centers that have a transplantation program, but many reports have indicated insufficient referrals in centers without LT. In 1992, Davies *et al*[32] reported that only one out of 42 patients with end-stage alcoholic cirrhosis admitted to a British district general hospital was referred to a transplant center. One of the explanations for this low referral rate was that a vast majority of the alcoholic patients were not abstinent upon admittance, which was considered a mandatory criterion at the time. However, there was also a lack of acknowledgment that LT was a therapeutic option for end-stage liver disease in general, as attested by the under referrals for other etiologies. A more recent study in a large Veterans Affairs medical center in the United States showed similar results. Only 21% of the 199 patients with potential indications for LT (according to the American Association for the Study of Liver Disease guidelines) were referred for a pre-transplant evaluation. The determinants associated with a negative mention of LT were old age, member of a colored population, and ALD[33].

Most transplantation teams require a 6-mo delay for abstinence. However, the validity of this criterion in predicting post-transplant relapse and prognosis has only been suggested and never convincingly demonstrated[34-37]. A more pragmatic attitude is to refer the patient regardless of their abstinence and allow the transplant team to judge the need for transplantation.

PRE-LT ASSESSMENT

Like all other LT candidates, patients suffering from ALD must undergo a thorough assessment to detect potential contraindications. Tissues at risk from alcohol damage, such as the heart, kidneys, immune system, and central and peripheral nervous system, must be carefully examined. A retrospective study from the United Kingdom highlights the importance of the pre-LT evaluation. Anand *et al*[38] examined data from their selection protocol from 1987-1994, where 180 patients with ALD were referred for LT. Out of the 137 patients with complete information, only 31% received transplants or were awaiting LT; almost 10% died during the evaluation time period, 14% were considered too healthy for transplantation, and 5% refused LT. The remaining 40% of patients were considered too ill, either medically or psychologically unsuitable for the procedure. Although it has been shown that *de novo* cancer mortality increases after LT in alcoholic patients (as discussed below), there is a lack of specific studies examining the approach to improve the detection of patients at risk of malignancies.

MANAGEMENT OF ALCOHOL ADDICTION BEFORE LT

Before LT, alcohol abuse and dependence must be evaluated by an addiction specialist using well-established diagnostic criteria, such as the DSM-IV[39]. Published data are scarce with regards to addiction management during the waiting-list period. It is common practice to ask patients to sign a contract to remain abstinent and encourage attendance at support groups such as Alcoholic Anonymous. A randomized controlled trial conducted by Weinrieb *et al*[40] in two United States centers compared Motivational Enhancement Therapy (MET), a positive reinforcement technique, with referral to local treatment sources ("treatment as usual"). While the study revealed that 25% of the patients drank alcohol before their transplant, MET had little, if any, influence on this event. By contrast, an Italian single-center retrospective report emphasized the importance of having a team of addiction specialists and close follow-up of the patients on the waiting list. After the creation of an Alcohol Addiction Unit within the LT center at the Gemelli Hospital in Rome, post-LT relapse rates decreased significantly. Follow-up of the patients on the LT waiting list or those under consideration for inclusion to the list occurred on a weekly basis during the first month, every other week during the second and third month, and finally every month[41].

MORTALITY AFTER LT

The graft and patient survival rates of ALD-related LT have consistently improved over time and are now at least as good as other LT indications. The latest reports from large databases such as the ELTR, which includes data from over 89000 LT, has revealed 1-, 5-, and 10-year patient survival rates of 86%, 73%, and 59%, respectively[6]. These rates are similar to those seen in the United States[42] and in exclusive living-donor liver transplantation (LDLT) in Japan and South Korea[43,44]. However, when the primary indication for LT is both ALD and chronic HCV infection, the survival rates decrease significantly[9,28]. As of now, it is unclear whether the latest and highly active antiviral drugs

Table 1 Summary of the incidence of alcohol relapse after liver transplantation in alcoholic liver disease patients from various studies since 2000

First author (country, yr)	Number of patients	Mean follow-up (mo)	Definition of relapse	Relapse rate	Impact on survival rate
Berlakovich (Austria, 2000[92])	118	53.7 (9-179)	Any consumption	13%	NA
Burra (Italy, 2000[93])	51	40.1 (0-86)	Any consumption	33%	NA
			Occasional drinking	21%	
			Heavy drinking	12%	
Tomé (Spain, 2002[76])	68	38 (12-68)	Any consumption	10%	NA
			> 60 g/d	3%	
Cuadrado (Spain, 2005[69])	54	99.2 (14-155)	Intake > 30 g/d	25.90%	Yes
De Gottardi (Switzerland and France, 2007[37])	387	61.1 ± 47.5	Intake > 40 g/d	11.90%	No
Karim (Canada, 2010[52])	80	NA	Daily intake or associated with medical harm	10%	NA
Dimartini (United States, 2010[66])	265	NA	Any consumption	48%	Yes
			Fluctuating low level	28.60%	
			Early onset rapidly accelerating moderate use	6.40%	
			Steady increase to moderate use after three years post-LT	7.40%	
			Early onset continuously increasing heavy use	5.80%	
Faure (France, 2012[70])	206	81.7 (29-135)	Any consumption	43.70%	Yes
			Slip	7.00%	
			Occasional intake (< 20-30 g/d)	12.40%	
			Excessive intake (> 20-30 g/d)	24.30%	
Egawa (Japan, 2014[44])	140	43.4 (0.1-163)	Any consumption	22.90%	Yes

NA: Not available.

directed against the hepatitis C virus will have any impact on this situation.

The causes of mortality differ between patients who receive a transplant for ALD and patients with other indications. Indeed, cancer (especially of the aerodigestive tract) and cardiovascular-related deaths are over-represented in the ALD group, according to European and United States registries[9,45]. These data, along with other reports[45,46], suggest that smoking has a direct influence on the mortality of ALD patients after LT, although this issue has not been extensively studied. DiMartini *et al*[47] published a prospective study that included 172 ALD-related LT recipients and focused on the effect of tobacco consumption after the procedure. They found that the smoking prevalence fluctuated from 39%-58% at different time points after the LT, the amount of daily consumption increased over time, and patients quickly become nicotine dependent.

RELAPSE

The first team to report a favorable result for alcoholic patients who underwent an LT was initially optimistic because of their low relapse rates (*i.e.*, only one out of 35 patients who lived for six months or longer relapsed). Starzl *et al*[14] referred to LT as "the ultimate sobering experience". While the goal is to have a relapse rate of zero, and reports showing up with up to 46% of patients returning to some kind of alcohol consumption after LT, the transplant community has since been more cautious. Table 1 summarizes the recent data for relapse rates after LT for ALD patients.

Definition

The variability found between reports is, in part, explained by the definition of relapse used. Indeed, it is important to differentiate between those patients who have minor, irregular, and occasional drinks (called "slips"), those who have a regular but moderate alcohol intake, and those who return to major and harmful drinking. In other words, the term relapse, as it relates to drinking, can differ from relapse to alcoholism[48]. Although minor, "controlled" consumption is unlikely to lead to graft damage, forgoing abstinence, regardless of the frequency or amount of alcohol consumed, is usually considered a relapse. This tenet is supported by a large longitudinal cohort study led by Vaillant who showed that "controlled" drinking cannot be sustained for periods longer than three years before returning to alcohol abuse, which can harm the liver graft[49].

Pre-transplant risk factors

Numerous reports have tried to predict patients at risk of relapse, using the pre-transplant patient characteristics and addiction, psychiatric, medical, personal, and family history. While some of the data are contradictory, the following factors have been identified in patients who relapsed after their LT: < six months of sobriety pre-LT[34,37]; family history of alcoholism[50]; psychiatric comorbidities, including other substance abuse[37,50,51]; diagnosis of alcohol dependence[51]; prior alcohol rehabilitation[51]; and female gender[52].

The high risk alcoholism relapse (HRAR) scale is a clinical score developed to predict the risk of relapse

among veterans[53] according to their daily alcohol consumption, length of their drinking history, and previous treatment history. While this scale showed that the six-month abstinence criterion alone would penalize a significant number of candidates who had a low relapse risk based on their HRAR score[54], it proved inefficient, when used by itself, to distinguish between those who relapsed and those who did not in a United States transplant population[55]. In a Franco-Swiss cohort of 387 patients who underwent LT for alcoholic cirrhosis, a HRAR score > 3 was one of the three factors associated with a relapse to harmful drinking (defined by a declared alcoholic consumption level > 40 g/d and the presence of alcohol-related damage), along with < six months of abstinence and presence of psychiatric comorbidities. The absence of all of these factors resulted in a 5% relapse rate, which increased to 18%, 64%, and 100% when one, two, or three factors were identified, respectively. It is noteworthy to point out that the vast majority (94%) of the cohort had less than two risk factors[37].

Detection

A patient's reliability regarding their alcohol history can be problematic, especially before transplantation, because candor can have negative consequences on their disease management (*e.g.*, waiting-list withdrawal, no re-transplantation). Yates *et al*[56] interviewed 50 patients with alcoholic cirrhosis and compared their results with those from an interview with an unbiased source who was unaware of the patient's alcoholism history. They found a good correlation between the two versions, concluding that patients suffering from ALD were reliable. By contrast, random blood alcohol level tests performed on patients who were on the waiting-list revealed hidden consumption in two studies[57,58]. After transplantation, several methods have been tested to identify relapsing patients, including histological findings[16,59], patient and entourage interviews[17,60], and blood and/or urine analyses[61,62]. Our transplant center[63], like others[64], uses a multi-detection method, combining clinical, biochemical, urinary and blood alcohol levels, and histological findings. This seems to be the most relevant method to detect alcohol relapse after LT[65].

Consequences of relapse

DiMartini *et al*[66] prospectively followed 208 patients who received a LT for ALD and characterized five different trajectories of behavior towards alcohol after the surgery. Over half of the patients (54%) did not consume any alcohol (group 1). Non-abstainers were divided into four categories: fluctuating low level of use (group 2, 26%), early moderate use that diminished over time (group 3, 6%), late moderate use that increased over time (group 4, 7%), and early heavy use that increased over time (group 5, 6%). Five deaths occurred in the study, which were all attributed to recurrent ALD. Interestingly, these patients were only from the early relapse groups (groups 3 and 5). These patients had more biopsy-diagnosed steatohepatitis and acute rejection than the other patients and were more prone to graft failure. In another study, Rice *et al*[67] showed that continuous heavy drinking is a risk factor of graft loss.

A relapse to harmful drinking seems to have an impact on the survival rates when it is assessed ten years after the LT[68,69]. Interestingly, Faure *et al*[70] revealed the negative impact of excessive alcohol consumption on survival after an LT irrespective of the primary indication. This emphasizes the importance of detecting this behavior in every LT recipient.

ALCOHOLIC HEPATITIS

Among all of the complications associated with ALD, acute alcoholic hepatitis is one of the deadliest. The standard of care includes 28-d oral steroid therapy for the severe forms of the disease, which are defined by a Maddrey's discriminant function > 32[71]. Response to this therapy is defined by a seven-day Lille score < 0.45. The six-month mortality is approximately 15% for patients who respond to therapy; it raises to over 75% for non-respondents[72]. These patients were initially excluded from transplantation programs because their condition implies a lack of abstinence. Based on pathological analyses of the excised liver, some reports have suggested a good outcome after LT; however, these studies did not include pre-LT histological proof of alcoholic hepatitis, and the patients were not prioritized according to the severity of their disease[73-75], except in one study[76].

In 2011, a French and Belgian group, led by Mathurin[77], conducted a trial that included 26 highly select patients suffering from biopsy-proven severe acute alcoholic hepatitis without response to corticosteroids who were listed shortly after the assessment of non-response. After receiving an early LT, the patients' six-month survival increased dramatically to 77%; the control group had a six-month survival of 30%. Only three of the patients resumed drinking, and none experienced graft dysfunction. The inclusion criteria for the early LT were: severe alcoholic hepatitis as the first liver-decompensating event, presence of close supportive family members, absence of severe coexisting or psychiatric disorders, and patient agreement to adhere to lifelong total alcohol abstinence. These stringent selection criteria resulted in less than 2% inclusion of patients in the study. Despite the new wave of controversy caused by this study[78], many transplant programs decided to accept patients for LT under these conditions[79]. A clinical trial is currently being conducted to confirm the low relapse rates observed[80].

OTHER PARTICULAR FEATURES OF ALD PATIENTS AFTER LT

Malignancy risk

It is well established that patients who receive a liver transplant have a 2-3-fold increased risk of developing solid organ cancer; this climbs to 10-30-fold for developing post-transplant lymphoproliferative disorders (PTLD) and skin cancers, when compared to general population[81-84]. Since the 1990's, several reports have determined that the risk of malignancy for PTLD and solid organ cancer was higher among patients who received a transplant for ALD[45,46,85,86]. The survival rates are heavily impacted by the occurrence of a non-skin malignancy[45]. Upper aerodigestive squamous and lung carcinomas are overrepresented in the ALD population after LT, suggesting there is an influence of tobacco consumption. It is unclear whether a relapse in harmful drinking also increases the malignancy risk.

Compliance and rejection rates

Thirty years ago, it was commonly believed that ALD patients would have difficulties following the stringent restrictions required by the immunosuppressive drugs, which would cause low compliance and high rejection rates; however, the situation is more nuanced than this. Indeed, patient compliance is high overall, similar to that found for other indications[9,59]. Furthermore, data from the 1990's, when tacrolimus was not as widely used as it is now, suggest that ALD patients present lower rates of acute cellular rejection[21,87]. A relapse to drinking can have two types of consequences for liver rejection. If the relapse is accompanied by a significant reduction in the intake of immunosuppressive drugs, there is an increase in the acute and chronic rejection rates[63]. By contrast, a return to drinking without discontinuation of medication can lead to lower rejection rates[88], probably from the effects of alcohol-related immunosuppression.

Neurological complications and depression

The neurological system can be damaged by excessive alcohol consumption, which can manifest in both the central and peripheral nervous systems. Buis *et al*[89] compared the resulting neurological complications following LT between patients transplanted for ALD or HCV as the primary indication. They found that the ALD patients experienced acute confusion for three or more days more often than the HCV patients (48% *vs* 6%, respectively, $P < 0.0001$). A shorter duration of sobriety before the LT was a risk factor, and the acute confusion experienced by the ALD patients caused a longer hospital stay. Additionally, ALD increases the risk of posterior reversible encephalopathy syndrome, although this remains an uncommon event[90].

Finally, in 2011, DiMartini *et al*[91] reported a prospective study that examined depressive symptoms in ALD patients after LT. They found three different trajectories of depressive symptoms that evolved within the first year after LT: consistently low depression levels (group 1), initial low depression levels that rose over time (group 2), and high depression levels at all time points (group 3). Interestingly, the ten-year survival rates differed significantly between group 1 (66%) and the other two groups (46% and 43% for groups 2 and 3, respectively). It is still unknown whether the type of depression management employed has an impact because the study was not designed to address this particular matter.

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TOPIC HIGHLIGHT

2015 Advances in Liver Transplantation

Current status and perspectives in split liver transplantation

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Abstract

Growing experience with the liver splitting technique and favorable results equivalent to those of whole liver transplant have led to wider application of split liver transplantation (SLT) for adult and pediatric recipients in the last decade. Conversely, SLT for two adult recipients remains a challenging surgical procedure and outcomes have yet to improve. Differences in organ shortages together with religious and ethical issues related to cadaveric organ donation have had an impact on the worldwide distribution of SLT. Despite technical refinements and a better understanding of the complex liver anatomy, SLT remains a technically and logistically demanding surgical procedure. This article reviews the surgical and clinical advances in this field of liver transplantation focusing on the role of SLT and the issues that may lead a further expansion of this complex surgical procedure.

Key words: Liver transplantation; Split liver; Segmental liver; Organ shortage; Graft sharing; Waiting list; *In situ*; *Ex vivo*; Allocation policy

Core tip: The transplantation community has made numerous efforts to expand the donor pool. While conventional split liver transplantation in which a child received the left lateral segment and an adult the right liver has proved an effective approach to increase organ availability, current outcomes after split liver transplantation for two adult recipients are conflicting. Ongoing surgical refinements and innovations have been reported and dedicated organ allocation policies proposed to encourage the more widespread application of this challenging procedure in the future.

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INTRODUCTION

More than a quarter of century since the first cases were performed, split liver transplantation (SLT) remains one of the few surgical options to expand the donor pool in view of the ongoing shortage of organs and the increasing waiting list mortality rates.

Reduced-size liver transplantation has already been introduced to address the shortage of small donors, and surgical reduction of donor livers to treat small children has been performed successfully in several centers[1-3]. This option improved the allocation of livers without any increase in the organ supply discarding the removed part of the liver. The year 1988 saw the clinical application of the split liver procedure and the first attempts to expand the donor pool.

In February 1988, Pichlmayr's team in Hannover demonstrated that one donor liver could be transplanted in two recipients by splitting the liver along the umbilical scissure in such a way that the left part (segments Ⅱ and Ⅲ) could be transplanted into a child, and the right part (segments Ⅰ, Ⅳ, Ⅴ to Ⅷ) into an adult[4]. In the following months, other authors reported their initial experiences with this innovative surgical technique splitting the liver by an *ex vivo* dissection of the vascular structures and parenchymal transection. Bismuth and colleagues[5] from the Paul Brousse Hospital, reported an emergency orthotopic liver transplantation in two adult recipients with fulminant hepatic failure. Using one liver divided along the main scissure, they shared the right hemiliver (segments Ⅴ-Ⅷ) and left hemiliver (segments Ⅱ-Ⅳ) while the caudate lobe was resected. Both recipients recovered from coma and regained normal liver function, however both died within two months after transplant from causes not specifically related to the operative technique.

Emond *et al*[6] from the Chicago group reported their preliminary experience with SLT describing a different splitting procedure with technical details related to recipient and donor size. Outcomes demonstrated the feasibility of the procedure highlighting the advantages of SLT in the pediatric population and advocating its role in adult recipients. In the same year, the team at Saint-Luc University Hospital in Brussels, Belgium described the surgical technique applied in the first two cases of SLT at their Institution[7]. Later, many single-center series of *ex situ* SLT were reported from Europe and the United States reflecting efforts to encourage wider application of this surgical technique in clinical practice[8-15]. These initial experiences included a high proportion of high-risk patients in both the adult and pediatric recipient cohorts especially in the main American series.

Based on the experience gained with living donor liver transplantation (LDLT), further expansion of this complex surgical procedure was pioneered by the Hamburg group in 1996 and by the UCLA group in 1997. They first reported a detailed description of the liver splitting procedure in a heart-beating deceased donor - the so-called "*in situ*" split liver technique - instead of the *ex vivo* procedure, using the technique described for left lateral live donor liver procurement[16,17]. Both teams claimed the *in situ* procedure provides superior results, mainly related to shorter cold ischemic time (CIT) avoiding prolonged bench surgery, and long-distance graft sharing between pediatric and adult liver transplant centers.

The first Asian SLT program started in Taiwan in 1997 after SLT had been performed in the other major liver transplant centers in the region with expertise in LDLT[18].

Splitting one liver between two adult recipients was the other goal to achieve to optimize the use of cadaver donors. After the initial attempts to transplant two adult recipients with one liver reported by the Bismuth group, other authors adopted this challenging surgical technique[5]. Two new surgical splitting techniques to transplant two adult recipients were proposed by Colledan *et al*[19] in 1999 and by the Hôpital Beaujon group in 2000 with detailed descriptions of the two surgical techniques[20]. Other small series subsequently demonstrated the feasibility of the procedure, reporting technical refinements and long-term outcomes after SLT for two adults.

The last decade has seen widespread application of SLT for adult and pediatric recipients as a result of the increasing experience of splitting techniques, and numbers are expected to increase in the near future. A similar evolution is to be expected in SLT for two adults especially in high-volume experienced hepatopancreatobiliary and transplant centers.

This article reviews the current status of SLT focusing on surgical technique, outcomes, and other clinical and logistical aspects regarding organ allocation policy and graft sharing.

TERMINOLOGY AND DEFINITIONS

There are two main types of SLT. The universally accepted definition of "conventional" split liver divides the liver to achieve a right extended graft (REG) (Couinaud segments Ⅰ, Ⅳ-Ⅷ) and a left lateral graft (LLG) (Couinaud segments Ⅱ and Ⅲ) for one adult and one pediatric recipient (A/P SLT). The split liver technique for two adult recipients (A/A SLT) divides the liver along Cantlie's line resulting in one right graft (RG) (Couinaud segments Ⅴ-Ⅷ) and one left graft (LG) (Couinaud segments Ⅰ-Ⅳ). Since it was first introduced, different definitions have been proposed for this surgical option including true-right/left split and

full right/full left SLT[21-24].

In addition, two surgical techniques can be applied to split a liver. The "*ex vivo*" procedure splits the liver after a standard multi-organ procurement and the parenchyma and vessels are dissected on a back table with the graft in an ice bath. Conversely, when hilar dissection and parenchymal transection are performed in a heart-beating deceased donor before procurement in a manner similar to LDLT, the technique is named "*in situ*" split.

SNAPSHOT OF SLT WORLDWIDE

Liver splitting is technically challenging and may increase morbidity and mortality. Despite the promising results reported in the last few years, SLT for two adult recipients has remained relatively uncommon, and no more than two hundred transplants have been performed worldwide. Conversely, conventional SLT has long been an established practice but its worldwide expansion differs widely.

European centers have been more active than those in other regions, and alternative procedures to whole liver transplantation (WLT) have been increasingly used in recent years: despite differences across countries in the rate of SLT, about 6% of all LT used split liver grafts[25]. Indeed, a 2006 report by the North Italian Transplant program (NITp) set a more than 20% split rate in a five-year period[26]. By contrast, SLT comprised only about 1% of all LT in the United States, and only 288 SLT were performed in adults between 2002 and 2009 despite estimates that approximately 20% of all deceased donors meet UNOS guidelines for split livers[27]. The reasons for such disparity are probably related to two main challenges in SLT such as graft allocation and recipient selection.

LT in Latin America is currently performed in 13 countries, and is growing heterogeneously despite a limited pool of available organs. Transplant programs from the largest countries of the region have continuously involved LT for almost two decades including LDLT, and reduced, partial, split, dual graft, and domino liver transplantation[28]. According to the 2013 report from the International Registry in Organ Donation, 35 of the 323 LT performed in Argentina were SLT accounting for about 10%[29].

Different reports from the Arab World have described a recent evolution in LT to pediatric transplantation and split liver techniques, and four split liver procedures were recently performed in Saudi Arabia[30,31].

Efforts to explore this surgical technique have been made in Africa and 14 cases of SLT in pediatric recipients were recently reported from a South African transplant center[32].

Split liver grafts continue to make a significant contribution to the total number of LT performed in Oceania, providing 223 (6%) of 3728 grafts by the end of 2013 in adult recipients[33].

Despite extensive experience in LDLT and liver resection, the extreme scarcity of cadaveric liver donors in Asia adversely affects the expansion of SLT. Nevertheless, the Taiwan group demonstrated the feasibility of SLT for two adult recipients in the MELD era performing 21 split liver procedures[18,34-36].

ANATOMICAL CONSIDERATIONS

«La race humaine veut des idées simples. Or, le réel est compliqué» - C. Couinaud.

The high incidence of surgical complications could not only be related to inherent technical failures but probably to an incomplete understanding of the segmental surgical anatomy of the liver, namely bile duct anatomy, the intrahepatic venous drainage, and the vascularization of segment Ⅳ. Some peculiar features of liver anatomy require a thorough understanding to perform split liver procedures.

In general, the left liver shows a more constant anatomy compared to the complex right lobe. Therefore, the more you stay on the left, the fewer the anatomical variations you will encounter. More than 50 years later, the anatomical studies performed by Couinaud remain of paramount importance in current practice[37]. Regardless of the type of split live procedure performed, biliary anatomy is one of the most demanding issues in SLT.

In 2000, Dr. Emond[38], one of the pioneers of SLT, published a comprehensive clinicopathological study investigating the liver anatomy applied to SLT. Anatomical data from *ex vivo* analysis of human liver casts were correlated to in vivo data from partial liver transplants performed in their initial experience. Four specific patterns of left biliary anatomy and three patterns of left hepatic venous drainage were identified and described. The study focused on the left biliary system, identifying a left bile duct plate at the junction of the ducts from segments Ⅱ and Ⅲ, and specific anatomical patterns were described. From the study' s anatomical considerations, when a conventional split liver procedure is performed, the dissection plane would have to be maintained one centimeter lateral to the umbilical fissure in segment Ⅳ to have a 90% chance of a single duct from segments Ⅱ and Ⅲ. Surgical considerations on this issue and the need for two different biliary anastomoses when transplanting a segment Ⅱ and Ⅲ graft have been reported by other authors[17,39].

Almost all centers experienced in LDLT have published benchmark studies investigating the consequences of venous anatomy for the split liver surgeon[40,41]. Different surgical techniques to assure optimal venous drainage will be mentioned in another chapter.

A precise knowledge of segment Ⅳ anatomy is of paramount importance when approaching split liver procedures regardless of the type of graft procured as adult recipients often suffer surgical complications

related to segment Ⅳ ischemia or impaired vascularization and biliary drainage. Many authors involved in this field of LT have called for special attention to be paid to the arterial supply to segment Ⅳ especially when the splitting line runs close to the falciform ligament removing and impairing all the portal branches to segment Ⅳ[27,42]. Another original article from the Korean experience describes in detail the anatomical variations of the origin of the segment Ⅳ hepatic artery and their surgical implications in SLT. As previously reported by Couinaud, Jin *et al*[43] highlighted some interesting aspects of hepatic embryology related to the complex segmental liver anatomy.

SPLIT PROCEDURE: TECHNICAL ASPECTS AND SURGICAL REFINEMENTS

Little has changed in the surgical technique adopted for conventional SLT since the first cases described, whereas different surgical refinements have been proposed to the technique first adopted in A/A SLT for both donor and recipient.

Although many authors advocated leaving the celiac trunk to the LLG in A/P SLT to give a surgical advantage to the pediatric population, certain anatomical situations in both donor and recipient should be discussed on a case-by-case basis and the surgical technique adapted accordingly[9,44,45].

As widely reported in adult LDLT, the venous outflow of the right graft and the addressing of the MHV remain controversial in SLT for two adult recipients. In recent years, many authors have reported their experience and proposed different algorithms based mainly on the dominance of one of the hepatic veins on imaging studies, graft-to-recipient weight ratio (GRWR) and remnant liver volume[46].

In March 2000, the group from Hôpital Beaujon, Paris Ⅶ University, Clichy, France described a modification of the *in situ* splitting technique consisting in a transection performed along the main portal scissure retaining the MHV with the left graft[20]. In the same year, Gundlach *et al*[47] first described how to split the vena cava (the so-called "split cava technique") to provide an optimal venous drainage of both hemiliver grafts and to overcome the decision on addressing the MHV and the vena cava to the left or to the right graft. In two different donor procedures they performed liver transection and bile duct division *in situ* while the vena cava was divided by a longitudinal transection of the front and back walls on the back table after division of the right hepatic artery and right portal vein. The resulting two grafts, each with a large venous patch including both the main suprahepatic vein plus all the additional smaller veins draining directly into the vena cava were transplanted using the piggy-back technique with a side-to-side cavo-colostomy[48]. Although this surgical refinement should solve the disadvantages of both options on where to leave the MHV, the split cava technique was not widely applied in subsequent years.

In 2001, Andorno *et al*[49] reported the long-term results obtained in the first series of eight adult patients undergoing A/A SLT. In their initial experience, no impairment in venous outflow of the right hemiliver was observed leaving the MHV with the left hemiliver provided that the entire right accessory inferior hepatic vein (IHV), with a diameter greater than 5 mm, was reconstructed. Similarly, Azoulay *et al*[50] described a split liver graft preparation where the MHV was kept on the left in continuity with the common trunk of the left and middle hepatic veins. This was also undertaken by Humar *et al*[51] and Zamir *et al*[52] while reporting the first cases performed.

In 2002, Yersiz *et al*[53,54] from Dumont-UCLA Transplant Center, University of California, Los Angeles described details of two different *in situ* split procedures for the creation of split grafts suitable for two adult recipients. To create a left graft including segments Ⅱ, Ⅲ, Ⅳ and a right graft including segments Ⅰ, Ⅴ-Ⅷ, they preserve the common portal vein and the common hepatic duct with the right graft, while the celiac axis is preserved with the left graft in order to maximize the arterial supply to segment Ⅳ. A common cuff including MHV and the left hepatic vein (LHV) is divided from the vena cava and retained with the left graft. The same group also described a different technique for the creation of a larger left graft including segment Ⅰ. The right hepatic vein (RHV) is identified and any accessory IHV larger than 5 mm in diameter is preserved. Before parenchymal transection, the left bile duct is sharply transected at the hilar plate. An isolated Pringle maneuver of the left hilar structures is performed to create a line of demarcation where the transection line runs along the main portal fissure. All the major venous branches draining segments Ⅴ and Ⅷ are preserved for later perfusion and revascularization. The parenchymal transaction is completed at the level of the inferior vena cava (IVC). After cold perfusion, the right portal vein, and the right hepatic artery (RHA) are divided just distal to the bifurcation, and the RHV is divided from the suprahepatic vena cava with a caval patch to complete the creation of a right segment Ⅴ through the Ⅷ graft.

Fan *et al*[34] reported the first case of an *ex vivo* A/A SLT performed at University of Hong Kong Medical Centre in 2003. Their technique consisted in a right lobe including the vena cava and the MHV with parenchymal transection to the left of the MHV. The patient transplanted with the left lobe suffered massive bleeding from the transection surface and congestion of segment Ⅳ and an arterial-portal regurgitation in segment Ⅳ, which finally became atrophied. In this case, the authors raised several concerns on the venous drainage of segment Ⅳ in the left lobe graft and segments Ⅴ and Ⅷ in the right lobe graft. This phenomenon seems to be similar to the remnant liver

of a living donor who had undergone an extended right lobectomy including the MHV[55]. However, the development of arterial regurgitation and venous collateral formation may be impaired in SLT due to the additional preservation-reperfusion injury.

In 2004, Humar *et al*[51,56] modified the splitting technique previously reported, and advocated several advantages in preserving the vena cava with the right graft. Indeed, by preserving the IVC with the right lobe, all short hepatic veins draining the right lobe are kept intact, and all the major hepatic tributaries to the MHV can be reconstructed on the back table improving the venous outflow, minimizing warm ischemic time thereby resulting in a less technically demanding and time-consuming procedure.

Conversely, in the same year Hwang *et al*[57] integrated their surgical knowledge from hundreds of adult LDLT into the first successful A/A SLT performed at the Asan Medical Center, Seoul, Korea transplanting a left lobe (segments Ⅰ to Ⅳ) with the vena cava, common bile duct, and celiac trunk. They advocate thorough planning of the splitting procedure with donor liver size assessment by CT scan before donor surgery as one of the essential steps as in LDLT, and hepatic venous anatomy evaluation especially in routing the MHV.

Again in 2004, Broering *et al*[58] from the Hamburg group published a review discussing the anatomical and technical aspects applied to SLT and summarizing their experience of both conventional SLT and A/A SLT. For conventional SLT, they reported different tips and tricks concerning the anatomic situation after dissection of the portal branches to segment Ⅳ, exposure of the left hilar plate behind the left portal vein, and dissection of the bile duct(s) from segments Ⅱ and Ⅲ. In A/A SLT, their practice is to retain the MHV with the left graft and the vena cava with the right graft as the division of the veins draining segment Ⅰ lead to uncertain viability of the caudate lobe that may require resection. Different strategies to provide optimal venous drainage of both hemiliver grafts were reported including the cava split and other venous reconstructions. Lastly, they discussed the merits of intraoperative cholangiography to identify anatomical bile duct variations, and to decide whether to leave the common bile duct to the left or right grafts. Individual donor arterial anatomy - especially the origin of the segment Ⅳ artery - should govern the sharing of the arterial trunk in both conventional and A/A SLT.

A retrospective study of our experience reported in 2008 pointed out some interesting surgical aspects related to our initial series of 16 *in situ* A/A SLT[59]. As for living donor liver surgery, the resection line was defined both by the parenchymal demarcation obtained after clamping the right hepatic artery and right portal vein, and by intraoperative ultrasound assessment of the MHV course leaving the MHV to the left graft[60].

As for LDLT, the inclusion of the MHV with one or the other graft remains controversial. In 2005, Broering *et al*[61] first described this challenging surgical option to optimize the outflow in both the full right and full left grafts in A/A SLT. They reported the first two livers split according to this new technique for *ex vivo* splitting. After dissection of the hilar structures and opening the vena cava in the midplane, the MHV was split in the middle from its orifice in the vena cava. After completion of the liver parenchyma transection, the two halves of the MHV were reconstructed using donor iliac vein patches.

In 2009, Chakravarty *et al*[62] remarked on the significance of caudate lobe outflow reconstruction in A/A SLT left lobe recipients as previously reported by others in LDLT[63]. They proposed the routine reconstruction of caudate lobe veins greater than 3 mm in diameter to preserve graft volume and function.

Recently, Lee *et al*[36] from the Taiwan group reported some important technical measures applied to A/A SLT when liver grafts were thick and the IVC might be compressed after graft implantation. In this circumstance, the left hemiliver graft (including the MHV) was turned over and implanted in the right liver fossa. A longitudinal incision was made on the IVC and the conference orifice of the middle and left hepatic veins of the graft was anastomosed directly to the IVC.

Heaton *et al*[64], another pioneer in this field of liver transplantation, proposed a technical strategy to expand graft availability by combining the established techniques of conventional SLT and use of the dual graft technique pioneered by Lee *et al*[65]. He suggests using two LLS grafts from two conventional split donor procedures performed simultaneously or by combining a LLG from a living donor and a conventional split liver graft from a deceased donor[64]. The advantages are significantly lower morbidity and mortality for the LLS living donor and a satisfactory liver volume for the adult recipient, improving outcome and reducing the risk of small-for-size syndrome. This strategy is technically demanding and requires surgical skills and significant infrastructure, logistical, and organizational changes but could lead to a potential increase in the number of adult transplants in the United Kingdom of 15%-20% per year.

A recently reported creative solution to the organ shortage is the option of performing sequential or domino liver transplantation with split livers from patients with familial amyloidotic polyneuropathy[66-68]. This uncommon surgical procedure had led to three transplants and represents the maximum example of organ sharing with a true domino effect: the combination of SLT using a cadaveric graft and sequential transplantation using a living whole liver donor.

Issues related to graft size are of paramount importance in partial liver transplantation and especially in A/A SLT. Generally speaking, an estimated GRWR of 0.8% or more is considered a reference in adult to adult LDLT[69]. According to recently published data on a large cohort of A/A SLT from Taiwan, it is

better to allocate split liver grafts to recipients with GRWR greater than 1%[36].

In the authors' experience, a comparison of clinical profiles between recipients of RL grafts and LL grafts showed that low GRWR was the only significant difference for the LL graft recipients and the small-for-size syndrome was common when transplanting LL graft. Although SLT often lacks the pre-operative imaging essential for a liver volume estimation due to logistic and administrative limitations, a precise estimation of liver mass remains crucial. The same authors reported a simply and accurate method to evaluate liver mass using bedside liver ultrasonography and standard liver volumes as an alternative to measuring hemiliver graft sizes.

In situ vs ex vivo SLT

The choice to split a liver *in situ* or *ex vivo* deserves special mention. The advantages of both these options have been widely discussed over the years since the introduction of the *in situ* procedure[16,17]. Data from a national survey published in 2004 demonstrated comparable results for the two different surgical methods in terms of morbidity and mortality except for a higher rate of post-operative bleeding after *ex vivo* SLT, confirming the feasibility of both splitting techniques[70]. A paper published in 2011 by Vagefi *et al*[22] described a large single-center experience with SLT performed from 1993 to 2010 comparing outcomes of *in situ vs ex vivo* split liver grafts emphasizing operative technique and surgical morbidity. They reported no significant differences in survival between adult recipients of grafts split *ex vivo vs in situ* or complication rates. More recently, the same authors retrospectively analyzed nine true right/left *ex vivo* split liver procedures performed during the same period and demonstrated that excellent long-term patient and graft survival can be obtained in A/A SLT with the *ex vivo* option[71]. From a surgical point of view, the *in situ* procedure abolishes *ex vivo* benching and prolonged ischemia times, allowing a better definition of the transection plane and providing two grafts with hemostasis accomplished. In addition, performing intraoperative ultrasound and vascular clamping during the parenchymal transection provides a better evaluation of venous drainage especially for A/A SLT while vascular and biliary evaluation during the *ex vivo* procedure is accomplished using angiography, cholangiography, or the instillation of dilute methylene blue[71].

Lee *et al*[36] recently described a modified *in situ* technique where the liver was split as much as possible during the donor operation but completed after perfusion with preservation solution. In the authors' practice, the bile ducts were divided before cold perfusion for a better understanding of the correct cut point while the parenchyma transection was completed quickly *in situ* with the liver surrounded by ice after procurement of thoracic organs in order to save time for other organ recovery teams.

However, the *in situ* procedure requires a longer operative time that should be expected in the setting of a multivisceral procurement especially in the presence of some degree of hemodynamic instability. The choice of the preferred technique should take into account the habits and experience of the surgical team, donor characteristics and logistic considerations, as well as the allocation policy applied in sharing the second graft. Indeed, it is the wider application of SLT, regardless of the technique preferred by the center, that will result in the largest number of split grafts benefitting the most recipients.

SPLITTABLE DONOR

Which donor livers are splittable? Clearly, the ideal donor suitable for splitting is young with no history of liver disease, normal liver enzymes, hemodynamically stable, and with a short hospital stay. Different criteria for donor splitting have been proposed in recent years and vary among countries and transplant centers[26,72-75]. A recently published report from a specific multicenter SLT program established in 1997 by the NITp listed the following donor eligibility criteria for the split procedure: age less than 60 years, intensive care unit stays shorter than five days, low inotropic support (dopamine $\leqslant$ 5 μg/kg per minute, dobutamine $\leqslant$ 10 μg/kg per minute, and no epinephrine or norepinephrine), and near-normal liver function tests[76].

While criteria for a conventional split liver procedure have been extended in recent years with adjustments to many parameters such as donor age and organ quality, donor requirements remain more pronounced if an A/A SLT procedure is planned[74]. Although donor parameters are critical for selecting livers for splitting procedures, defining absolute contraindications to splitting is difficult and donors should be evaluated on a case-by-case basis after *in situ* evaluation of the liver by an experienced surgeon.

When splitting a liver for two adult recipients, other issues play an important role in the decision whether to split or not to split. The body weight and clinical status of the potential recipient, as well as the availability of an experienced surgeon, and a number of logistical considerations have to be evaluated. While an *in situ* conventional split procedure can be done in any hospital, and no specialized equipment is required, logistical aspects may play a crucial role in planning a split procedure to create grafts for two adult recipients where preoperative imaging evaluation of the liver anatomy and volume may advance donor-to-recipient match and graft allocation. An algorithm for the "real-time" matching of donors and recipients on the waiting lists was recently reported in a multicenter Italian study. The algorithm is based on the GRWR

and graft sharing considering a liver suitable for an A/ASLT procedure whenever no pediatric recipients are available[77].

OUTCOMES

The majority of published series have compared the outcomes of SLT and WLT. We report the outcomes after split liver transplantation from different series considering the different type of graft transplanted. In addition, some special aspects related to the surgical technique and other important issues such as allocation policy, donor and recipient selection, and logistical considerations published in the last ten years are also discussed.

The results of conventional SLT are equivalent to those of whole liver transplantation when performed by experienced groups, and SLT has become a standard procedure in pediatric liver transplant centers.

In 2006, a matched pair analysis by the Hamburg group compared long-term results after extended right SLT and WLT in adults, confirming no differences in patient and graft survival rates[45]. These findings were confirmed in a matched pair analysis by another experienced group from Bergamo[78] and in other single-center reports[79,80].

Results from another large-volume transplant center further confirmed equivalent long-term graft survival rates in both adults and children for segmental grafts with those in WLT. Hong *et al*[81] from the UCLA Transplant Center reported a single-center analysis of 2988 LT performed between August 1993 and May 2006 with a median follow-up of five years. Split-liver grafts included 109 left lateral and 72 extended right partial livers while 49 left lateral and 41 right grafts (segments Ⅴ-Ⅷ with MHV inclusion) from living donors were performed. The ten-year patient survival rates for WLT, SLT, and LDLT were 72%, 69%, and 83%, respectively (P = 0.11), while graft survival rates were 62%, 55%, and 65%, respectively (P = 0.088). Comparing outcomes between adults and children separately by graft types, the adult ten-year patient survival rate was significantly lower for split extended right liver graft compared with adult whole liver and living-donor right liver graft (57% *vs* 72% *vs* 75%, respectively, P = 0.03), while graft survival for adults was similar for all graft types. Conversely, in children, the ten-year patient and graft survival rates were similar for all graft types. Although ten-year graft survival rates after WLT, SLT, and LDLT were comparable in adults, the patient survival was lower for split grafts compared with whole grafts when used in retransplants and critically ill recipients. Interestingly, the authors proposed an alternative system to allow optimal use of split grafts in the current MELD system. In the algorithm proposed, when a donor meets the split criteria proposed by Toso *et al*[72] and the LLG is allocated, the REG instead is matched to an ideal recipient by the splitting transplant center rather than through the MELD system. According to the authors, an organ allocation system with such flexibility would encourage adult-to-child candidate pairing by the same transplantation center and allow preoperative surgical and logistic planning to minimize graft ischemia duration. This proposal aims to optimize graft-to-recipient matching that not only would substantially reduce the loss of lives on the transplant waiting list but also improve outcomes after liver transplantation.

In 2009, Cescon *et al*[21] from the University of Bologna group raised some important considerations regarding recipient selection (donor/recipient match) as a critical aspect of SLT in adult recipients especially in centers implementing a MELD-based allocation policy. They reported the outcomes of 22 *in situ* SLT performed in five years from 2003 comprising both A/A SLT and conventional SLT (2 RG, 3 LG, 11 ERG, ad 6 LLG) in adult recipients. A flexible donor procedure was proposed and the choice how to split was related to donor liver size, and to recipient size and clinical conditions on the basis of the harvesting surgeon's judgment. Recipients with higher MELD scores received right grafts, while smaller adults with no or mild portal hypertension were given left grafts. Overall patient and graft survival rates were 90% and 86% respectively. Patient survival was 84% in recipients of right grafts and 100% in recipients of left grafts. Graft survival was 84% and 89%, respectively. Vascular and biliary complications occurred in 14% and 4% of cases. The authors claimed that LLS should not be excluded a priori for a small adult, and SLT for two adult recipients can be successfully performed even using left lateral segments by assigning one graft according to the MELD score, with a more liberal allocation of the second graft.

In 2012, Zambelli *et al*[24] reported a retrospective analysis of an Italian multicenter experience including long-term results after A/A SLT and graft sharing between November 1998 and January 2005. Their data concerned 43 A/A SLT performed by five centers with more than 60% of grafts shared among centers. According to the Clavien[82] classification, 31 (72%) had complications above grade Ⅱ while three (6.9%) were retransplanted. Hospital mortality was 23% and sepsis was the main cause of death. Actuarial survival rates at one and ten years were 72.1%, 62.6% and 65.1%, 57.9%, respectively for patients and grafts, similar to those reported for adult LDLT by the European Registry over a similar period[83]. The authors emphasized the importance of their multicenter collaboration especially in graft sharing in order to overcome organizational limits and increase the application of this complex procedure.

Another approach to expand the donor pool has been the use of donation after circulatory death (DCD) donors, and these now represent approximately 20 per cent of the cadaveric liver transplant activity in the United Kingdom[84]. Interestingly, Mallik *et al*[85] retrospectively compared outcomes after 17 *ex vivo*

adult SLT (extended right grafts) from donors after brain death (DBD) and 32 WLT from "controlled" donors after DCD (Maastricht category Ⅲ donors) performed at the Cambridge Transplant Centre between January 2004 and December 2010[86]. No formal guidelines exist as to which segment the common hepatic artery and aortic patch are preserved and this is usually left for discussion between the adult and pediatric centers.

None of the 32 patients in the DCD cohort suffered early graft failure, compared with five of 17 in the ERL-DBD series. Reasons for graft failure were hepatic artery thrombosis (HAT) in three cases, progressive cholestasis, and a small-for-size syndrome. In the DCD group, ischemic cholangiopathy developed in six patients, resulting in graft failure within the first year in two, whereas the other recipients remained stable. The incidence of biliary anastomotic complications was similar in both groups. Kaplan-Meier survival analysis confirmed superior graft survival in the DCD liver group (93% at three years *vs* 71% in the ERL-DBD cohort, $P = 0.047$), comparable to that of the remaining 426 whole DBD liver transplants (93% at three years). Patient survival was similar in all groups. According to the authors, one of the reasons possibly related to the poorer outcome in the RL-DBD cohort was the unavoidable much longer CIT due to the splitting procedure and the time required to transport these graft to different centers. As reported elsewhere, we believe that scrupulous recipient selection and an aggressive approach to minimize CIT by considering *in situ* rather than *ex vivo* splitting may improve outcomes with SLT from DBD donors[87].

In 2013, Doyle *et al*[88] demonstrated equivalent outcomes between SLT and WLT reporting the results from a single center retrospective analysis investigating 53 recipients receiving SLT out of 1261 (4.2%) transplants performed from 1995 to 2012. Interestingly, they advocated the use of intraoperative cholangiography to identify a suitable biliary anatomy for splitting and described why they commonly leave the celiac axis with the left lateral segment graft. Twenty-three adults received split grafts: 18 (78%) were right trisegment grafts, four (17.4%) were right lobes, and one (4.3%) was a left lobe. The rates of patient and graft survival at one, five and ten years in adult recipients of split grafts were 95.5%, 89.5%, and 89.5%, respectively. Survival was similar to that of whole organ recipients ($P = 0.15$). Thirty children received split grafts. At one, five and ten years, pediatric split overall and graft survival rates were 96.7%, 80.0%, 80.0%, and 93.3%, 76.8, and 76.8%, respectively ($P = 0.81$). Complications included three retransplantations (10.0%), five bile leaks (16.7%), two cases of HAT (6.7%), two bowel perforations (6.7%), and two bleeds (6.7%). Once again, the authors concluded calling for collaborative networks to be established to maximize liver splitting and consolidate suitable organ allocation.

Very recently, Lee *et al*[36] from the Taiwan group examined the outcomes of A/A SLT in the MELD era, reporting comparable results with those of LDLT even in patients with high MELD scores. Forty-two patients who underwent *in situ* A/A SLT (21 RG and 21 LG) were compared to 282 adult patients who underwent LDLT performed in the period between 2003 and 2010. In a MELD-based allocation policy one of the grafts was allocated to the first priority patient in the waiting list with the highest MELD score while the other was allocated to a size-matched recipient. The MHV was preserved to the left lobe while the IVC was preserved to the right lobe. The large tributary veins (> 5 mm in diameter) of segments Ⅴ and Ⅷ were reconstructed with venous grafts and drained into the IVC. Among 42 A/A SLT recipients, 24 (57.1%) had MELD scores higher than 20. The median (interquartile) MELD score was significantly higher than that for the recipients with LDLT ($P < 0.001$). The complication rates for right or left hemiliver allograft transplantation did not differ ($P = 0.213$), nor did the overall survival rate ($P = 0.457$). The survival rates for SLT at one, five and ten years were comparable with those of LDLT ($P = 0.489$).

These findings were confirmed by the Hannover group after a case by case evaluation of their series of cases performed in the MELD era. In the authors large experience the survival of patients with MELD score greater than 30 at time of SLT were not worse as compared to recipient with a lower MELD[89].

Once again, Hashimoto *et al*[90] reported favorable outcomes after A/A SLT when a MELD system regulates organ allocation. In a 9-years review of their experience the Cleveland group report outcomes of 25 grafts (10 left lobes and 15 right lobes) transplanted in adult sized recipients between 2004 and 2012. Split graft recipients experienced biliary complications more frequently (32% *vs* 10.7%, $P = 0.01$); however, the 5-years graft survival for split grafts was comparable to WLT (80% *vs* 81.5%, $P = 0.43$).

Aseni *et al*[77] compared the outcomes of 64 recipients of A/A SLT prospectively selected using a computerized algorithm in the NITp over a 12-year period among seven collaborative centers with WLT performed in the same period. They described in detail the value of the algorithm developed for "real-time" matching of donors and recipients on the waiting lists on the basis of calculated GRWR and graft sharing considering a liver suitable for AASLT whenever no pediatric recipients are available. The retransplantation rate (9.2%) after A/ASLT was similar to the 10.2% in the WLT group and to the European and United States liver retransplantation figures. The one- and five-year patient and graft survival rates with A/ASLT were significantly lower than for the WLT control group. The five-year graft survival rate of 58.3% for A/A SLT seems closer to the 56% reported in other high-risk liver graft recipients using "marginal donors" or "cardiac death" donors[83]. According to the type of split liver graft, five-year survival rates for patients receiving full

left grafts or full right grafts were 67.2% and 59.3% for patients and 60.7% and 56.6% for grafts but the differences were not significant.

One- and five-year survival rates for the 64 AASLT were 73.2% and 63.3% for patients, and 63.3% and 58.7% for grafts. One- and five-year survival rates for the 1199 patients who received WLT in the same period were 87.2% and 83.1% for patients and 85.2% and 80.4% for grafts. Outcomes were significantly different, with better survival rates in the WLT group ($P = 0.0003$ for patients and $P < 0.0001$ for grafts).

Cauley *et al*[91] recently aimed to determine the current risk of graft failure in adult recipients after SLT. They analyzed data from UNOS concerning 889 split live grafts performed from 1995 to 2010. Similarly to previous analyses from the United States, the authors noted a significantly increased risk of graft failure in split grafts compared with whole grafts in the pre-MELD era from 1995 up to March 1, 2002 when the MELD score was first introduced. Conversely, the risk of graft failure was similar between SLT and WLT recipients in the most recent MELD era with a split-liver hazard ratio of 1.10 ($P = 0.28$) in the MELD era (2002-2010).

Queen Elizabeth Hospital group from Birmingham, United Kingdom, first systematically analyzed SLT outcomes from a technical reconstruction point of view comparing 171 adult right lobe SLT procedures and 1412 WLT procedures performed between January 2000 and June 2012[74]. They described different vascular and biliary reconstruction options in detail, analyzing specific surgical complications against reconstruction techniques. The overall incidence of vascular and biliary complications in the SLT group was greater that in the WLT group ($P = 0.009$ and $P = 0.001$, respectively) whereas no survival difference between the two groups was reported. Overall patient survival rates at one, three and five years were 83%, 80%, and 76% for SLT patients and 86%, 81%, and 77% for WLT patients (0.58). Graft survival was 79% *vs* 83%, 76% *vs* 78%, and 72% *vs* 74% at one, three and five years for SLT and WLT patients, respectively ($P = 0.45$). Their findings indicate that multiple hepatic arteries supplying a right lobe graft were probably related to a higher risk of early graft loss from HAT, although any option of arterial reconstruction using the RHA of the graft combined with a direct biliary anastomosis may result in an increased incidence of biliary complications.

Our experience

By the end of 2014, 1763 LT had been performed at our institution, the Niguarda Hospital Cà Granda, Milan, including 178 segmental liver grafts. We started to expand the donor pool using SLT in 1996. Seventy-one adult recipients underwent conventional A/P SLT and 19 A/A SLT, while since March 2001 (initiation of our LDLT program) 88 adult LDLT have been performed. Except for the first four cases of conventional A/P SLT, the splitting procedures were performed *in situ*. A detailed description of the surgical technique adopted at our center, the algorithm applied for donor selection, and the split-liver allocation policy have been described elsewhere together with a detailed analysis of morbidity[26,59,77]. Concerning A/A SLT, some technical adaptations have been implemented thanks to the growing experience in LDLT and liver surgery such as the use of different surgical devices for parenchymal transection, the addition of intraoperative ultrasound and the mandatory application of a radiological anatomical evaluation before donor surgery.

Patient and graft survival rates at one, five and ten years after conventional A/P SLT were 88.2%, 79.2%, and 68.8%, and 85%, 77.4%, and 69.3% respectively. According to the Clavien classification of surgical complications, 12.7% (9/71) of patients experienced grade 4a complications leading to retransplantation, 7% (5/71) grade 3b complications, and 2.8% (2/71) grade 3a complications.

Patient and graft survival rates at one, five and ten years after A/A SLT were 73.7%, 73.7%, and 67%, and 73.7%, 68%, and 68% respectively. Five patients (26.3%) experienced grade 5 complications (one anastomotic bile leak, one HAT, one hepatic vein thrombosis, and two sepsis) leading to death, one (5.26%) a grade 4a complication (HAT) leading to retransplantation, and four (21%) grade 3b complications with a complete recovery after surgical treatment. The outcomes of our single-center series compare favorably with the overall outcomes reported by others and recently published.

FUTURE PERSPECTIVES

Favorable results with SLT depend not only on the technical factors described over the years but also on scrupulous recipient and donor selection, and dedicated resources. The need to expand the donor pool has justified perseverance in improving the surgical technique after the initial experience with the conventional procedure that led to the current good results. Transplanting two adult patients with one cadaveric liver is the ultimate way of meeting the liver organ shortage without the risks associated with using a living donor. Although A/A SLT still carries a relatively high risk of surgical complications and failure, it is our hope that it will become an established routine in the future. Past failures will help us to understand and define the circumstances under which this type of transplant can be safely performed and how to avoid some of the more frequent complications unique to this procedure.

Close cooperation among centers with adequate experience in split liver techniques is mandatory and should be encouraged. SLT often lacks the preoperative imaging essential for a liver mass estimation and anatomical evaluation. Administrative

limitations must be overcome to accommodate these imaging requirements before donor surgery and organ allocation in order to advance the best graft-to-recipient match. Improving allocation policies by better patient and donor selection plays a crucial role in SLT, and "ad hoc" algorithms for donor-to-recipient matching should be developed and widely applied. Dedicated resources and incentives must be made available to implement programs and facilitate surgeon recruitment and training even though current data do not yet fully justify the investment. In the words of Professor Henry Bismuth, "the highest risk for a patient needing a new liver is the risk never to be transplanted".

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TOPIC HIGHLIGHT

2015 Advances in Liver Transplantation

Clinical significance of donor-specific human leukocyte antigen antibodies in liver transplantation

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Abstract

Antibody-mediated rejection (AMR) caused by donor-specific anti-human leukocyte antigen antibodies (DSA) is widely accepted to be a risk factor for decreased graft survival after kidney transplantation. This entity also plays a pathogenic role in other solid organ transplants as it appears to be an increasingly common cause of heart graft dysfunction and an emerging issue in lung transplantation. In contrast, the liver appears relatively resistant to DSA-mediated injury. This "immune-tolerance" liver property has been sustained by a low rate of liver graft loss in patients with preformed DSA and by the intrinsic liver characteristics that favor the absorption and elimination of DSA; however, alloantibody-mediated adverse consequences are increasingly being recognized, and several cases of acute AMR after ABO-compatible liver transplant (LT) have been reported. Furthermore, the availability of new solid-phase assays, allowing the detection of low titers of DSA and the refinement of objective diagnostic criteria for AMR in solid organ transplants and particularly in LT, have improved the recognition and management of this entity. A cost-effective strategy of DSA monitoring, avoidance of class Ⅱ human leukocyte antigen mismatching, judicious immunosuppression attached to a higher level of clinical suspicion of AMR, particularly in cases unresponsive to conventional anti-rejection therapy, can allow a rational approach to this threat.

Key words: Donor-specific anti-human leukocyte antigen antibodies; Liver transplantation; Rejection; Acute antibody-mediated rejection; C4d; Solid-phase immunoassays; Human leukocyte antigen single antigen bead

Core tip: The role of donor-specific anti-human leukocyte antigen antibodies (DSA) in liver transplant (LT) remains unclear. Alloantibody-mediated adverse consequences are increasingly being recognized, and several cases of acute antibody-mediated rejection after ABO-compatible LT have been reported. There is a need to investigate and quantify the potential adverse impact of DSA on LT outcomes. The present review addresses the current knowledge on this issue.

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INTRODUCTION

Although human leukocyte antigen (HLA) antibodies (Abs) have been more extensively studied in kidney transplantation, they can be detected after any solid organ transplantation. As with renal transplantation, the presence of anti-HLA Abs in heart and lung transplants is associated with a worse graft survival[1]. The impact of donor-specific anti-HLA antibodies (DSA) on short- and long-term liver transplant (LT) outcome is not clearly defined. In LT, the presence of preformed DSA is well recognized, although in most cases, DSA disappear a few months after liver transplantation. In the setting of DSA persistence and evidence of complement activation after LT, no significant clinical impact in the first year post-transplantation has been described[2]; however, recent reports indicate that some LT recipients who develop *de novo* DSA result in lower graft survival and patient survival[3-7]. Thus, there is a need to investigate and quantify the potential adverse impact of DSA on LT outcomes. The present review addresses the current knowledge on this issue with a particular focus on LT.

IMPORTANCE OF ANTIBODY-MEDIATED REJECTION IN SOLID ORGAN TRANSPLANTATION

The detrimental effects of DSA on renal transplantation outcomes have been recognized since 1969[8], and since then, strong evidence has indicated longer kidney allograft survival among patients without DSA. In this setting, the incidence of hyperacute rejection caused by pre-existing DSA has been nearly eliminated by performing a complement-dependent cytotoxic cross-match prior to kidney transplantation; however, acute and chronic antibody-mediated rejection (AMR) plays an increasingly critical role in kidney allograft loss and is considered among the most important barrier that limits long-term outcomes[9-14]. In 2003, at the National Institutes of Health conference, acute AMR in renal transplantation was defined as an acute rejection with graft dysfunction, histological evidence of acute tissue injury and C4d deposition in the presence of DSA[15].

The negative impact of alloantibodies directed against donor HLA antigens was subsequently widely demonstrated and accepted not only in kidney but also in heart transplant, and recent evidence also endorses this notion in pancreatic and lung transplantation[16-24]. For instance, whereas the incidence and mortality of cardiac acute cellular rejection (ACR) have decreased in recent years as a result of advances in immunosuppression, the incidence of AMR appears to be increasing[25]. Furthermore, AMR also seems to be an increasingly common cause of graft dysfunction and cardiac allograft vasculopathy[26,27]. In fact, the presence of DSA in these types of solid organ transplant may contraindicate the transplant due to the increased risk of acute rejection and lower graft survival[28-30]. Moreover, in these patients the development of *de novo* DSA after transplantation has also been associated with an increased risk of rejection and lower survival[22,24,31,32]. As a consequence of the above-mentioned problems, different strategies-from prevention, DSA monitoring, and selection of adequate immunosuppressive regimens to therapeutic approaches-have been adopted to minimize the deleterious effects of AMR. In the next sections we will focus on these factors.

ANTIBODY-MEDIATED REJECTION IN LIVER TRANSPLANTATION

Human liver allografts are highly resistant to acute AMR from preformed human HLA alloantibodies in comparison with kidney allografts[33]. In LT, the presence of preformed DSA is well recognized, although in most cases, DSA disappear a few months after liver transplantation. Several separate mechanisms in isolation or in combination have been postulated to explain this state of "immune privilege" in the LT setting[34,35]: (1) the liver secretes soluble HLA class I molecules that form immune complexes with alloantibodies, which are then cleared by Kupffer cells; (2) Kupffer cell phagocytosis of platelet aggregates and immune-complexes limits complement activation; (3) the limited distribution of HLA class Ⅱ expression in the microvasculature; (4) the great liver restorative and regenerative capacity before any insult, even mediated by the immune system; and (5) a large endothelial surface that is capable of absorbing circulating Abs. For example, in a rat model, DSA are cleared from the circulation in only 30 min when the serum is perfused through an extracorporeal liver of donor origin[36]. Other possible mechanisms proposed are

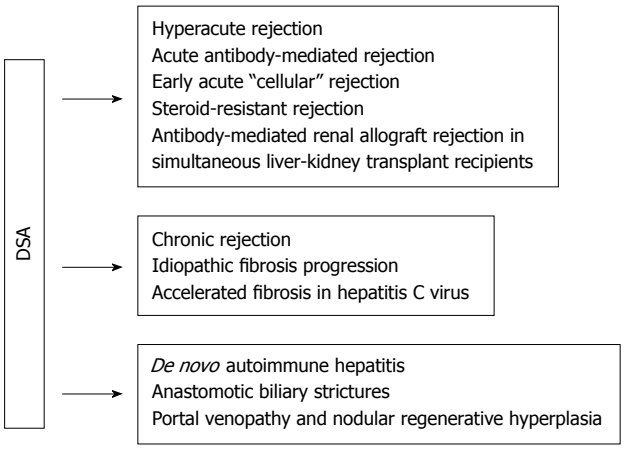

Figure 1 Potential associations of donor-specific human leukocyte antigen antibodies with outcomes in liver transplant or simultaneous liver-kidney transplant recipients. No associations have been confirmed in large randomized controlled trials. Adapted from O'Leary *et al*[34]. HLA: Human leukocyte antigen; DSA: Donor-specific anti-HLA antibodies.

related to the particular coagulation state in advanced liver diseases (the deficit of coagulation factors and thrombocytopenia-related portal hypertension can help reduce platelet aggregates and hence the formation of vascular thrombosis observed in humoral rejection mediated by DSA) that can facilitate the vascular flow, the hypocomplementemia of liver cirrhosis, and the dual hepatic vasculature that facilities improved flow during injury. This factor may decrease hepatic necrosis from arterial vasospasm and local intrahepatic coagulation that occur as result of DSA[34].

However, in the last years there have been different reports that highlight a potential deleterious role of preformed HLA Abs in liver graft survival[5,37-53]. Kozlowski *et al*[40] found that preformed DSA that persists after LT was associated with severe early rejection. Moreover, Krukemeyer *et al*[54] have revealed portal infiltration and proliferation of B lymphocytes (CD20) and plasma cells (CD138) as well as the expression of the B cell/plasma cell-activating chemokines MIP-3, CXCL9, CXCL10, CXCL11, and CXCL12 in acute liver allograft rejection. Recently, O' Leary *et al*[38] have found AMR to be a contributor to previously unexplained early liver allograft loss through the analysis of 60 patients with idiopathic early allograft loss when strict criteria for AMR diagnosis were fulfilled. The authors concluded that liver allograft recipients with preformed DSA with a high mean fluorescence intensity (MFI) seem to be at risk for clinically significant allograft injury and possibly for loss from AMR, often in combination with ACR. In addition, Musat *et al*[39] demonstrated that DSA is present in up to 75% of patients experiencing rejection, and both DSA and C4d staining was present in 54% of the patients diagnosed with ACR, demonstrating a previously unrecognized humoral component to these rejections. Furthermore, in this study 70% of the patients with ductopenia had DSA and 60% of the ductopenia cases had both circulating DSA in association with diffuse portal C4d deposition, supporting a role for AMR in the pathogenesis of interlobular bile duct injury and loss[39]. These results have been corroborated in other studies[31,37,40,51,55-59]. Morphometric studies have shown that portal tract microvasculature destruction precedes bile duct loss in the process of liver allograft rejection[38,57]. Thus, the following chain of events seems to occur: the formation of the DSA-HLA complex on endothelial cells of the portal tract microvasculature triggers complement activation (evidenced by C4d deposition) and destruction of the portal microvasculature/capillaries branching off the communicating artery from which the periductal vascular plexus arises[60], resulting in ischemic bile duct injury and loss. In fact, the resolution of cholestasis and ductopenia in association with a reduction of C4d deposition only after a decrease in circulating DSA with aggressive therapy specifically directed towards antibody removal further supports this role.

Certainly, no associations between donor-specific HLA alloantibodies with outcomes in liver or simultaneous liver-kidney transplant recipients (SLKT) have been demonstrated in large, randomized clinical trials[34]. Nonetheless, a panel of experts gathered in a recent meeting to discuss the different aspects regarding the consequences of DSA in liver transplantation agree that both acute AMR in liver transplantation recipients and an antibody-mediated renal allograft rejection observed in SLKT are two accepted associations on the basis of multiple case-control studies[34].

Regarding SLKT, "renal allograft protection" by the liver allograft occurs when the recipient harbors isolated preformed class Ⅰ DSA in low-to-moderate amounts[34]; however, inferior outcomes have been demonstrated when preformed high MFI class Ⅱ DSA is present[61,62]. In those cases, both the kidney and liver allografts are at a risk for rejection, especially when class Ⅱ DSA persists post-transplantation[62,63]. Patients who undergo SLKT should ideally receive organs without class Ⅱ antigens against which the recipient has DSA with an MFI > 5000.

Other potential associations described include the following: hyperacute rejection[64], *de novo* autoimmune hepatitis[65], anastomotic biliary strictures[66], and idiopathic fibrosis progression[60,67] (Figure 1).

DE NOVO DSA IN LIVER TRANSPLANTATION

The role of *de novo* DSA after LT remains unclear as the majority of studies have focused on preformed DSA. The risk of DSA development increases with a low immunosuppression load[60]. Infections and inflammatory events could alter the expression of class-Ⅰ and class-Ⅱ antigens and hence contribute to alloresponse induction and DSA development[68-70]. A recent report demonstrated that 8.1% of a cohort

Table 1 Association of graft fibrosis and concomitant anti-human leukocyte antigen class Ⅱ donor-specific anti-human leukocyte antigen antibodies

Ref.	No. of patients	Positive for HLA Abs	Transplant type	Follow-up. median (yr)	Time detection DSA	Method detection DSA	MFI
Miyagawa-Hayashino *et al*[78]	79	32	LD	11	After LT	SAB	> 5000
Salah *et al*[58]	114	5	LD	2	After LT	SAB	> 5000
O´Leary *et al*[60]	507	46	DD	6.4	Pre and after LT	SAB	> 5000
Grabhorn *et al*[72]	19	16	LD + DD	4.5	After LT	SAB	> 5000
Iacob *et al*[79]	174	34	LD + DD	ND	After LT	SAB	> 5000

HLA: Human leukocyte antigen; DSA: Donor-specific anti-HLA antibodies; SAB: Single-antigen-bead; MFI: Mean fluorescence intensity; LT: Liver transplant.

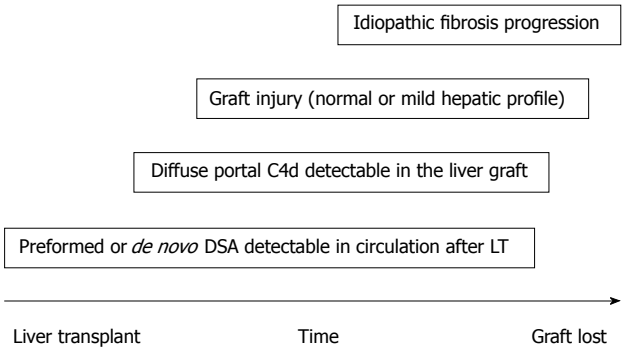

Figure 2 Idiopathic fibrosis progression. Hypothetical chain of events. DSA: Donor-specific anti-HLA antibodies; LT: Liver transplant.

of 749 LT recipients developed *de novo* DSA one year after transplantation (most of them against HLA-Ⅱ, especially HLA-DQ)[5]. *De novo* DSA resulted in lower graft and patient survival in a multivariate analysis. These findings were confirmed by Fontana *et al*[71] Moreover, 75% of the patients who developed *de novo* DSA had biliary complications. Furthermore, O'Leary *et al*[49] have shown the clinical relevance of *de novo*-specific antibodies on rejection and long-term survival. In addition, a higher rate of the *novo* DSA, especially of HLA-class-Ⅱ, in pediatric patients with chronic rejection has recently been observed[72].

IDIOPATHIC FIBROSIS PROGRESSION

Evidence has shown that the humoral alloresponse may have a role in interstitial fibrosis and tubular athropy development after kidney transplantation[73]. In LT, graft fibrosis is frequently observed in late biopsies from pediatric patients with a normal or mild hepatic profile, and the severity of fibrosis correlates with the timing from LT to biopsy[74-77]. Miyagawa-Hayashino *et al*[78] are the first to suggest a role of DSA and the humoral response in long-term fibrosis in LT. The LT patients with *de novo* DSA and normal graft function had a higher grade of fibrosis and inflammation with a C4d-positive biopsy than patients free of DSA. Importantly, this study showed an association between DSA and fibrosis, but the cause-effect was not demonstrated. Although other potential issues could explain the fibrosis such as subclinical biliary obstruction or venous flow, recent publications have confirmed the observations of Miyagawa-Hayashino (Table 1 and Figure 2)[58,72,79].

MECHANISMS OF ANTIBODY-MEDIATED REJECTION

The mechanisms involved in the DSA-mediated graft damage (inflammation, necrosis and fibrosis) can be summarized as follows[17-19]: (1) the complement activation by the classical pathway that induces complex formation of the membrane attack (indirectly detected using immunohistochemistry for C4d -a degradation product of C4, present at the site of complement activation- attached to vascular endothelium); (2) direct damage to the vascular endothelial capillaries through the interaction of the Abs to HLA and non-HLA antigens expressed on their cell surface; (3) platelet activation and aggregation causing the release of their granules containing growth factors, cytokines, chemokines and adhesion molecules that promote the recruitment and activation of pro-inflammatory cells; and (4) the DSA facilitate the activation of pro-inflammatory cells such as natural killer (NK) cells, macrophages and neutrophils, which express at their surface the receptor for the crystallizable fragment (Fc) of immunoglobulin (Figure 3). This cascade of events is morphologically translated by the observation of platelet aggregates, neutrophil accumulation, and microangiopathic thrombosis, causing cell necrosis and early graft failure. Chronic antibody-mediated rejection is due to repetitive thrombotic events and inflammatory phenomena culminating in fibrotic changes. The following pathological damages have been described after liver transplantation: platelet aggregates in the portal and/or centrilobular areas, neutrophil infiltration, patchy necrosis and centrilobular hepatocyte ballooning, cholangiolar proliferation, acute cholangiolitis and cholestasis[6,50,51,80].

DIAGNOSIS OF DSA-RELATED AMR

Because of the overwhelming evidence for antibody-

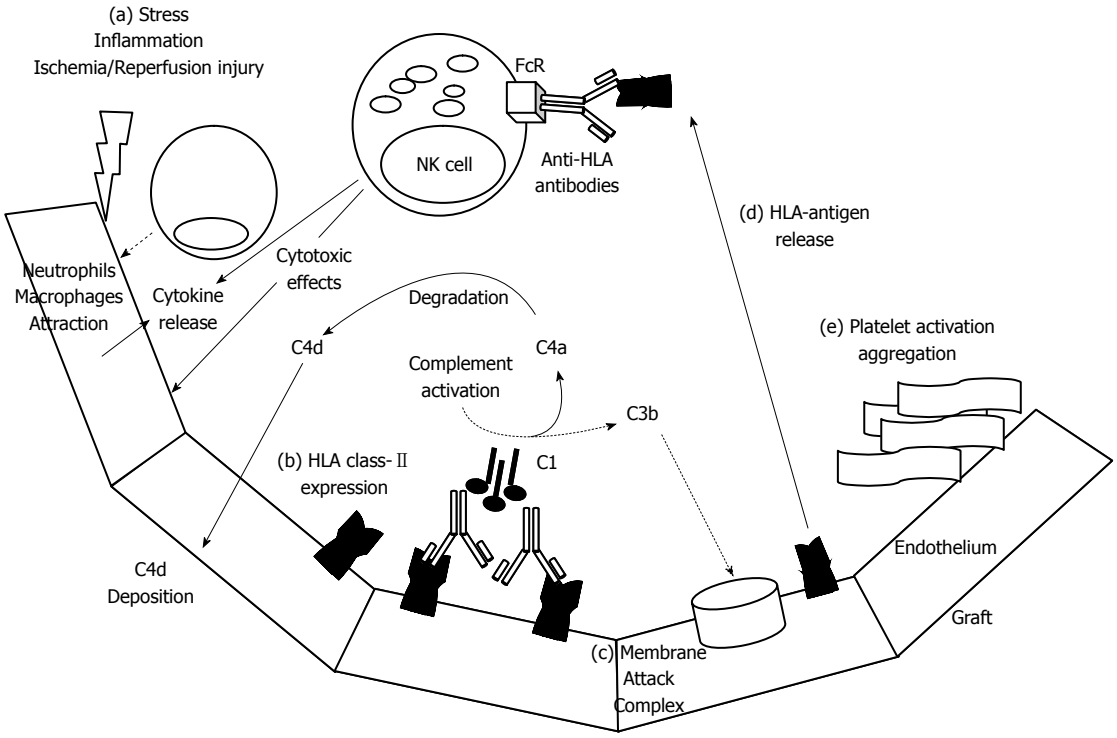

Figure 3 Mechanisms involved in humoral graft damage. Early post transplantation after ischemia/reperfusion injury (a) the endothelium can release several chemokines and cytokines to gather innate immune cells as neutrophils, macrophages. In this inflammatory setting, the graft endothelium could be activated and expressed human leukocyte antigen (HLA) class-Ⅱ antigens (b), subsequently, these antigens could be recognized by anti-HLA class-Ⅱ antibodies. If the antibodies are able to fix complement factors could trigger classical complement pathway that finally induce the membrane attack complex (c) on targeted endothelial cells. During complement activation, C4a component is degraded in C4d and finally deposited on capillaries. After destruction of endothelial cells, the HLA class-Ⅱ molecules could be released and directly detected by circulating anti-HLA antibodies that once recognized by FC receptors on NK cells could direct cytotoxic actions and cytokine production. Another potential mechanism of humoral graft damage could be driven by platelet activation and thrombi formation (e).

Table 2 Diagnostic criteria of acute antibody-mediated rejection in liver transplantation

The presence of DSA in serum
Histopathologic evidence of diffuse microvascular endothelial cell injury and microvasculitis
Strong and diffuse C4d positivity in tissue[1]
Reasonable exclusion of other causes of injury that might result in similar findings

[1]Diffuse portal microvascular positivity in formalin-fixed, paraffin-embedded samples (although detection of C4d is more sensitive in fresh tissue) is emerging as most strongly correlated with donor-specific anti-HLA antibodies-induced injury. DSA: Donor-specific anti-HLA antibodies.

mediated injury to kidney allografts, a consensus conference was held in 2003 to define the diagnostic criteria for antibody-mediated rejection in solid organ transplantation[15]. This group developed diagnostic criteria for AMR after kidney, heart or lung transplantation. Accordingly, the diagnosis of AMR requires clinical evidence of graft dysfunction, histologic evidence of tissue injury, immunopathologic evidence of an antibody response [complement component 4d (C4d) or immunoglobulin deposition] and serologic evidence of anti-HLA or anti-donor antibody at the time of biopsy.

In the setting of liver transplantation there are stringent criteria for the diagnosis of acute AMR that include the following (Table 2)[34,38]: (1) the presence of DSA in the serum; (2) histopathologic evidence of diffuse microvascular endothelial cell injury and microvasculitis; (3) strong and diffuse C4d positivity in the tissue; and (4) reasonable exclusion of other causes of injury that might result in similar findings.

Pre-transplantation cross-matching of the recipient's serum and the donor's lymphocytes has become a requirement of kidney transplant programs throughout the world on the basis of the known deleterious effects on kidney allografts of antibody-mediated graft injury[81]. In the setting of LT, there is a need to develop a cost-effective DSA monitoring algorithm, but a panel of experts has recently recommended a DSA monitoring schedule that includes testing all liver allograft recipients in the pre-transplant setting and, afterwards retesting all positive patients 1-2 wk post-transplantation to determine persistence[34]. There have been notable technological advances in the available assays to determine DSA. Earlier cell-based assays for DSA detection (*i.e.*, cytotoxic crossmatch) had several limitations in terms of sensitivity and specificity and the ability to differentiate between IgG from IgM Abs and between HLA from non-HLA Abs. Flow cytometry cross-matching is another cell-based assay that relies on the detection of Abs binding to the surface of donor

lymphocytes and is more sensitive than cytotoxic crossmatch. The first solid-phase immunoassay (SPI) used to test anti-HLA Abs was based on an enzyme-linked immune assay (ELISA), but recently SPI is being replaced by single-antigen-bead (SAB) assays. Acquired by Luminex™, this technology offers a new approach in the detection and quantification of post-transplantation anti-HLA Abs, which can be present in any solid transplant. This immunoassay allows the detection of low titters of HLA Abs that were undetectable by former assays, specifically and semiquantitatively[23,34,81]. The fluorescence signals detected are expressed as MFI or molecules of equivalent soluble fluorochrome (MESF). The isolated finding of HLA DSA is not specific for AMR because it has been found in 60% of LT recipients without rejection[37]. Certainly, most patients with preformed low-to-moderate levels of isolated class Ⅰ DSA in the absence of recurrent liver disease appear to have few, if any, short- or long-term consequences. Moreover, the significance of DSA late after liver transplantation without allograft dysfunction is uncertain[34]. As an isolated finding it does not represent an indication for intervention, although the long-term outcomes of such patients are thus far unknown.

C4d is a component of the complement cascade that is considered a marker of complement regulation. The complement system is a part of the innate immunological response and becomes activated in a variety of immunological events, such as ACR and viral and autoimmune hepatitis[82,83]. Different C4d staining patterns have been described in liver allografts. Even diffuse endothelial and sinusoidal C4d staining alone cannot be considered specific for the diagnosis of AMR as it has been found in AMR and other common allograft disorders such as ACR, chronic rejection, biliary obstruction and recurrent viral or autoimmune hepatitis[50,84]. Although there is no consensus, the diffuse portal microvascular positivity in formalin-fixed, paraffin-embedded samples (although detection of C4d is more sensitive in fresh tissue) is emerging to be most strongly correlated with DSA-induced injury[38-40,50,52,85]. Otherwise, C4d-negative AMR has been identified in renal allografts and likely occurs in the liver, although experts favor the above described conservative approach until more is learned about liver AMR[20,38].

Finally, the clinical presentation of liver allograft AMR is nonspecific, and many etiologies, such as ACR, ischemic injury, pharmacological toxicity, infections, initial graft dysfunction, hepatic artery thrombosis, biliary complications, and disease recurrence, can explain increases in aspartate aminotransferase (AST), alanine aminotransferase (ALT), and cholestasis[59,86,87]. AMR should be considered as part of the differential diagnosis if DSA are present. These observations have prompted the design of a multicenter study of specific features that could be used to screen patients for acute AMR *via* routine HE staining[88].

WHEN MUST AMR BE SUSPECTED?

Acute AMR occurs most commonly during the first several weeks after liver transplantation and consists of an otherwise unexplained liver allograft dysfunction associated with falling platelets and complement levels and increased levels of circulating immune complexes in patients with preformed, persistent DSA[67]. The liver biopsy shows microvascular injury in addition to other characteristics associated with allograft rejection, which is observed in approximately 1% of all early (< 90 d) liver allograft failures. Notwithstanding, acute AMR could explain up to 10% of idiopathic early liver allograft failures in DSA-positive patients[38].

Therefore, a high suspicion of DSA-induced AMR would theoretically be raised for a liver recipient with high titers of preformed anti-donor HLA class Ⅱ Abs who presents graft dysfunction in the early post-transplant period (first 90 d) that is otherwise not explained and is associated with falling platelets and complement levels and increased levels of circulating immune complexes. Furthermore, a negative response to conventional antirejection therapy is also associated[89]. SLKT recipients who receive crossmatch-positive organs are also the patients in which a high level of alert must be maintained, especially when the recipient has DSA with an MFI > 5000[62,63]; however, as stated above, there are other possible clinical presentations where DSA can play a pathogenic effect and thus could indicate the use of a diagnostic approach (*i.e.*, DSA assay, liver biopsy, *etc*.).

RISK FACTORS FOR DSA-RELATED AMR IN LIVER TRANSPLANT RECIPIENTS

Together with class Ⅱ HLA mismatching and prior cellular rejection, inadequate immunosuppression (particularly minimization and non-adherence to immunosuppressive medication) is a risk factor for the development of DSA[23].

Recognized risk factors favoring DSA-mediated liver damage were identified before the use of SAB technology allowed more accurate DSA determinations and included high-titer preformed Abs, the persistence of anti-donor Abs after transplantation, and otherwise unexplained thrombocytopenia and hypocomplementemia[38,51,65,90-92]. Thereafter, adverse outcomes have been associated with strongly positive flow cytometry cross-matches versus weakly positive cross-matches and strong preformed DSA evaluated for their complement fixing ability with a complement component 1q (C1q) assay[86]. C1q-binding DSA are expected to have the potential to assess cytotoxicity and have been associated with a greater risk of acute rejection and allograft lost in patients undergoing renal and heart transplantation[93-95]. Thus, in a recently proposed algorithm, a patient with strong DSA and C1q-positive DSA is considered at a higher risk and should be monitored for post-transplant DSA[59]. If

persistent DSA are detected, the patient is monitored as being at a higher risk for AMR.

Furthermore, the effects of DSA can vary depending on cofactors, some of which may promote immune stimulatory/profibrogenic effects and some of which could promote tolerogenic effects[34]. Thus, on the one hand, the up-regulation of DSA targets in allografts of patients with infections or inflammatory-mediated tissue damage[68-70] as occurs in patients with recurrent hepatitis C chronic infection, as a consequence, appears to be associated with fibrosis progression[60]. On the other hand, HLA class Ⅱ-restricted regulatory T cell (Treg) epitopes in IgG (also called "Tregitopes") that suppress immune responses to co-administered antigens may be formed as a result of DSA, thereby promoting tolerance[96].

PREVENTION AND MANAGEMENT OF LIVER DSA-RELATED AMR

As previously mentioned, the advent of new diagnostic technologies, particularly SAB assays, has allowed the assessment of the immunological risk in potential recipients of a particular donor by means of the identification and characterization of HLA Abs. In the kidney transplant setting, a detailed serological follow-up is of critical importance in the decision-making process because it can help determine whether to proceed with the transplantation, desensitize or follow a standard immunosuppressive (IS) therapy[23]. Efficient desensitization protocols have enabled successful transplantations, overcoming immunological barriers in patients including the barrier of a positive complement-dependent cytotoxic cross-match[97-99]. Anti-humoral therapy is based on two complementary approaches: (1) the removal of harmful Abs from the blood stream through plasmapheresis or immunoadsorption; and (2) the modulation of various components of specific and/or innate immunity using strategies including intravenous immunoglobulin, anti-CD20 antibody (rituximab), antithymocyte globulin (ATG), proteasome inhibitor (bortezomib), anti-C5 antibody (eculizumab), or even splenectomy[97-99].

In the setting of liver transplantation, the routine assessment of DSA pre-transplantation, with a retest of positive patients 1-2 wk post-transplantation, has been recommended by a panel of experts[34]. This fact is of particular interest when a SLKT is being considered and in the case of anti-donor HLA class Ⅱ Abs; however, there are several shortcomings with this strategy that need to be solved[34]: (1) only a small percentage of sensitized patients before transplantation will have severe, adverse consequences after transplantation; and (2) the significance of DSA late after liver transplantation without allograft dysfunction is uncertain and, in general, this finding does not merit any intervention. Taking into account these shortcomings, a panel of experts have recently proposed to investigate the design of cost-effective DSA monitoring strategies that allow one to detect the first group of patients and that identifies DSA characteristics late after transplantation that indicate inadequate immunosuppression or an unacceptable risk of chronic allograft injury[34].

Patients who undergo SLKT should ideally receive organs without class Ⅱ antigens against which the recipient has DSA with an MFI > 5000[34]; however, if a patient must receive cross-match positive organs after balancing the risks of a DSA-mediated rejection against those related to a protracted waiting list period in terms of progression of the liver disease, postoperative testing to determine antibody persistence and close follow-up are desirable[34].

Otherwise, the IS regimen and drug exposure can be relevant in terms of prevention of DSA-mediated allograft damage. In the kidney transplantation setting, the selection of an adequate IS can prevent subclinical inflammation and hence fibrosis progression[23]. For instance, in a case-control study, Moreso *et al*[100] confirmed the lower prevalence of subclinical inflammation associated with a regimen based on tacrolimus, mycophenolate mofetil, and prednisone than with a regimen based on cyclosporine, mycophenolate mofetil, and prednisone. In addition, lower exposure to tacrolimus between 3 and 12 mo after transplantation was independently associated with higher increases in chronic pathology in patients also treated with mycophenolate mofetil, and prednisone[101]. In the liver transplantation setting, *de novo* DSA prevention strategies also include a strict adherence to immunosuppression and the use of tacrolimus (rather than cyclosporine)[5,102,103].

The treatment of acute AMR in ABO-compatible liver transplants is not clearly determined because of the limited number of cases[34,104]. Most of the evidence in this field derives from studies in kidney transplantation where different anti-humoral therapies similar those mentioned above have been used. Bortezomib, a proteasome inhibitor effective in depleting plasma cells that in turn are responsible of producing the offending Abs, has been successfully used in three cases of severe AMR in ABO-compatible LT recipients[104]; however, concerns have been raised about the anti-humoral therapies in LT recipients because of their potent immunosuppressive effects that may exacerbate chronic viral hepatitis or increase infectious risks. Thus, experts currently advise that a strategy based on the combination of avoidance/prevention when possible may be the best strategy[34].

CONCLUSION

There has been a recent resurgence of interest in AMR in liver transplantation based on an increasingly number of reports indicating DSA-mediated allograft dysfunction and a better characterization of this entity in terms of diagnostic tools and diagnostic criteria.

Although AMR is a less frequent cause of liver allograft dysfunction, it must be taken into account not only from a diagnostic/therapeutic point of view but also from a preventive standpoint.

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TOPIC HIGHLIGHT

2015 Advances in Liver Transplantation

Candidates for liver transplantation with alcoholic liver disease: Psychosocial aspects

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Abstract

In Europe, 30% to 50% of liver transplantations are currently due to alcoholic liver disease (ALD). In the United States, this percentage is 17.2%. Post-transplant survival and other predictors of clinical course do not differ significantly from those in other types of transplanted patients, as long as there is no relapse of drinking. However, 20%-25% of these patients lapse or relapse to heavy drinking post-operatively, which has been associated with an increased risk of liver damage and mortality. It is therefore crucial to design specific selection and follow-up strategies aimed at this particular type of patient. Several good and poor prognosis factors that could help to predict a relapse have been suggested, among them the duration of abstinence, social support, a family history of alcoholism, abuse diagnosis versus alcohol dependence, non-acceptance of diagnosis related to alcohol use, presence of severe mental illness, non-adherence in a broad sense, number of years of alcoholism, and daily quantity of alcohol consumption. In this article, we discuss these and other, more controversial factors in selecting ALD patients for liver transplantation. Abstinence should be the main goal after transplantation in an ALD patient. In this article, we review the several definitions of post-transplant relapse, its monitoring and the psychopharmacological and psychotherapeutic treatment.

Key words: Liver transplantation; Alcoholic liver disease; Psychosocial assessment; Psychosocial selection

Core tip: Currently, alcoholic liver disease (ALD) is one of the most common indications for liver transplant, and post-transplantation survival and other predictors of clinical course in ALD patients do not differ significantly from other types of transplanted patients, as long as there is no relapse of drinking. It is crucial to design specific selection and follow-up strategies aimed at this particular type of patient. In this article, we discuss several factors that are important to consider

in the selection and follow-up of liver transplanted ALD patients.

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INTRODUCTION

One of the main indications for liver transplantation is alcoholic liver disease (ALD). In Europe, 30% to 50% of liver transplantations (LTX) are currently due to ALD[1], and this percentage is lower only than that due to liver disease caused by viruses[2]. In the United States, between 1988 and 2006, 17.2% of LTX were performed in ALD patients[2].

In the 1980s, based on studies of ALD patients who underwent LTX, the idea that this particular group of patients had a poor prognosis and clinical course became widespread[3-5]. Therefore, it was established that very strict criteria were needed to include ALD patients on liver transplantation lists (with the support of the United States National Institutes of Health)[6].

However, subsequent experience in transplantation has contradicted this evidence. It was concluded that the results of the earliest studies could be linked to the group of ALD patients in question presenting with important comorbidities that concurred with a poor prognosis[7]. Currently, post-transplantation survival and other predictors of clinical course (such as the length of post-transplantation hospitalization and number of rehospitalizations) do not differ significantly from those in other types of transplanted patients, as long as there is no relapse of drinking[8-10].

However, it is known that 20%-25% of these patients lapse or relapse post-operatively to heavy drinking[11], which has been associated with increased risks of liver damage and mortality, especially after several years of alcohol use[12,13].

Although LTX has become the treatment of choice for several types of liver disease, the need for organs largely exceeds their availability.

Therefore, in the social context of organ unavailability and the extremely high costs associated with the process of transplantation, it is paramount to select candidate patients according to certain criteria[14].

Due to social and moral factors, ALD patients are not always in the condition of to compete for transplantation under equal conditions with other patients[7].

This article aims at reviewing, based on the existing literature, the psychosocial aspects related to the selection, monitoring and follow-up of ALD patients subject to liver transplantation.

LITERATURE REVIEW

A literature review was conducted, for articles published between 1990 and 2015, in MEDLINE and PubMed using the following key words: "Alcohol Liver Disease", "Liver Transplantation", "Alcohol Liver Disease Treatment", and "Alcohol Liver Disease in Liver Transplantation". Three textbooks and 2 Web sites were also consulted. The 50 articles reviewed included clinical cases, reviews and original articles (observational studies, cross-sectional studies and observational, longitudinal studies).

RESULTS

Pre-liver transplant selection and relapse predictors

Given the complexity of the disease and transplantation processes, psychosocial evaluation must be incorporated into a more comprehensive consideration of the appropriateness of liver transplant for particular patients[11]. Several factors have been suggested to be predictors of relapse, with several scales being used for this purpose.

Factors that might determine relapse of drinking

Duration of abstinence: Pretransplant abstinence sometimes occurs in the context of the deterioration of the general condition of the patient, and it is sometimes precipitated by hospitalization; therefore, it should not be regarded as an absolute predictor of post-transplant abstinence[14].

In December 1996, the United Network for Organ Sharing recommended a minimum period of 6-mo pretransplant abstinence[7]. Several authors have criticized the use of such a short period of time to predict sustained and maintained abstinence[15]. Dew observed that a period of pre-transplant abstinence shorter than 6 mo could predict the rate of relapse of drinking[16]. Dom *et al*[17] calculated that, for every month increase in pre-LTX abstinence, there was a 5% decrease in the adjusted relapse rate.

Clinically, it has been believed that the imposition of a 6-mo period is not realistic, because some patients presenting for evaluation might not be able to survive 6 mo before being transplanted[18], as is the case with patients with acute alcoholic hepatitis, in whom the time frame is much shorter[17]. In contrast, 6 mo of abstinence might not be sufficient to allow some patients to recover their liver function, which might call into question the real need for a liver transplant[19].

Social support: Since the beginnings of pretransplant selection, psychiatrists such as Beresford and Vaillant have drawn attention to the importance of social support and social stability for good clinical and psychiatric post-transplant outcomes[20,21]. Dew and Kotylar considered social support to be one of the

most important factors in determining post-transplant alcohol use[16,22]. Having a stable partner and reliance on family or friends could also be positive factors, reducing the risk of relapse[17]. In contrast, a lack of social support and continued engagement in alcohol-related social activities could be negatively associated with relapse[17].

Other factors: Dew also drew attention to predictors of poor prognosis, such as a family history of alcoholism[16], and Kotylar emphasized a diagnosis related to alcohol use (better prognosis for alcohol abuse than for alcohol dependence)[22], which has been corroborated by recent studies conducted by authors such as DiMartini[23]. Also, a history of a severe mental disorder (a psychotic disorder -namely schizophrenia - or a personality disorder - namely antisocial personality disorder) and non-adherence in a broad sense (attendance of visits, compliance with treatment)[22] can be predictors of a poor prognosis. Yates emphasized the importance of the daily quantity of alcohol and the number of years of alcoholism. According to his model, the risk was greater for a number of drinks greater than 17 and a number of years of alcoholism greater than 25[24]. Additionally, non-acceptance of a diagnosis of alcohol dependence was found to be correlated negatively with post-LTX alcohol relapse[25].

Selection scales: Several psychosocial assessment instruments are used during the pretransplant period. Among these instruments, the Psychosocial Assessment of Candidates for Transplantation scale, with which aspects related to social support, mental health, lifestyle and understanding of transplant processes are rated[26], and the Transplant Evaluation Rating Scale, which assesses the degree of adaptation of the patient in distinct psychosocial and psychiatric areas: previous psychiatric history; current DSM-Ⅲ psychiatric diagnosis (axes Ⅰ and Ⅱ); substance use; compliance; healthy behaviors; social support; previous history of coping; current coping with the disease and treatment; affect; and mental state[27].

However, there are scales that specifically assess the eligibility of candidates with alcoholic liver disease. Among these scales, the High-Risk Alcoholism Relapse[24] scale and the Alcoholism Prognosis Scale[28] should be emphasized.

Moreover, some scales have also been shown to be useful in assessing other factors that might determine relapse of drinking. Among these scales is the Multidimensional Adherence Questionnaire, designed by Telles-Correia *et al*[29].

Controversial aspects in the selection of ALD candidates

Written contracts: In some transplant centers, the patient is required to sign a contract to formalize his or her acceptance of the diagnosis and the need for post-transplantation abstinence, as well as his her commitment not to relapse to drinking[20,30]. According to Beresford, this contract can help the doctor in cases of a post-transplantation relapse, and it might be a useful instrument to motivate the patient in seeking help[31]. However, the same author drew attention to the patients accepting diagnoses related to alcohol use in the pre-transplantation period not necessarily predicting their acceptance of the same diagnoses in the post-transplantation period. Therefore, the utility of this instrument might be uncertain.

Pre-transplantation assessment in patients with encephalopathy: The prevalence of hepatic encephalopathy in transplant candidates is high[32,33].

Therefore, it is only natural that many ALD candidates are encephalopathic at the time of pre-transplant assessment. According to Beresford, there are two problems in these cases: the quality of the assessment itself, which, given the cognitive limitations of the patient, has limited validity, and the utility of referring these patients (after assessment) to alcohol treatment programs if the degree of encephalopathy is maintained. In this way, whenever possible, the author advised that patients should improve their state before being submitted to transplantation[20]. If improvement is not possible, it might be useful to collect from the family, or a significant other, data from the anamnesis that are relevant to the transplant procedure.

Relapse occurring after listing: A relapse of drinking in the pre-transplantation period, after listing, is a very serious situation. Most centers have opted to exclude from transplant lists patients who, after having stopped drinking and having been accepted for transplant, relapse to drinking. Some centers, while not excluding the patients from the list, prefer to reassess them with particular strictness and to refer them for rehabilitation[20].

Definition of relapse and post-transplant psychological and psychiatric monitoring and follow-up of the ALD patient

Definition of relapse: The diagnosis of relapse depends on the definition used, and it is necessary to distinguish "relapse of alcoholism" (generally associated with the psychiatric diagnoses of alcohol dependence and abuse) and "relapse of drinking". In the context of liver transplant, "relapse of drinking" is mostly used, which assesses the presence of abstinence and quantified alcohol consumption.

The presence of abstinence must be the main objective after transplantation in an ALD patient[34], because alcohol use is associated with histological liver lesions, which develop rapidly and lead to fibrosis, so no consumption whatsoever should be allowed at this stage[32,35].

Along with the presence or absence of abstinence, frequency and intensity of consumption, translated into the number of drinks ingested, should also be assessed[24].

During the post-transplantation period, Kotylar distinguished "heavy relapse" from "slip". The former corresponds to the consumption of more than 5 drinks over a period of more than 5 consecutive days[22]. Some studies have reported that a "slip" indicates a much better clinical prognosis (particularly regarding survival rates) than a "heavy relapse"[36,37].

Relapse monitoring: Relapse monitoring can be undertaken using three strategies: self-reports; reports from family members; and laboratory tests.

The probability of the patient's self-reports being truthful is approximately 65% (compared to the reports from family members and the results of laboratory tests)[38].

As stated in other sections of the present article, family members can prove to be major allies, and their reports can be very useful in determining the patient' s alcohol consumption. For this process to occur, it is paramount that the transplantation team create a solid and transparent relationship with the patient's family[39].

Many centers resort to laboratory exams. Liver lesion tests can be useful for identifying patients who drink after a rehabilitation program, with sensitivity of 100% and a specificity of 82% when GGT $\geqslant$ 20%, ALT $\geqslant$ 40% and AST $\geqslant$ 20%[40]. However, these results are not valid in patients with significant liver disease[41].

Another test frequently used is the carbohydrate-deficient transferrin test. However, as mentioned, recent studies have shown the value of this test in the detection of prolonged alcohol consumption to be uncertain, especially in patients with very severe liver disease[42].

Alcohol quantification can be performed using urine, breath, blood and hair. While blood, breath and urine tests detect alcohol use in the previous 12-24 h (on average), hair tests can detect it up to 90 d before the exam[43].

Relapse treatment: Attitudes and behaviors toward treatment are relevant in the context of outcomes, but there have been few studies showing the efficacy of the psychosocial treatment of ALD patients after transplant. Several reasons have been noted for this situation, among them that the patients believe that they no longer have an alcohol-related problem and therefore no longer need treatment, as well as that certain drugs used to prevent relapse of drinking are feared by patients (and often also by their hepatologists) due to possible adverse reactions in patients without fully recovered liver function[22,44]. This finding explains why the only controlled, randomized study in this area was not completed[44].

Two types of treatment have been suggested: psychopharmacological and psychotherapeutic[2,45].

Regarding the former, 5 drugs should be emphasized.

Disulfiram inhibits the aldehyde dehydrogenase enzyme, leading to an accumulation of acetaldehyde (a compound that results from alcohol metabolism and is degraded by aldehyde dehydrogenase), which is very toxic to the organism, causing extreme vasodilation and the consequent drop in blood pressure, tachycardia and cephaleas. These effects are called "Antabuse" or "disulfiram-like". The use of this drug in transplanted patients is not recommended due to its potentially hepatotoxic and hypotension-inducing effects[44].

Naltrexone, an opioid receptor antagonist (especially of the μ receptor) is also widely used in alcoholism, having already been shown to reduce cravings and the rate of relapse of drinking[46]. Although its action is not yet clear, it seems to be mediated by the inhibition of the rewarding effects of alcohol, by blocking the reward circuits in the brain[47]. The administration of this drug in transplanted patients has been avoided because, according to some manuals, it can cause an increase in aminotransferases (which is generally reversible).

Nalmefene (the mechanism of which is similar to naltrexone but apparently with better liver tolerance) could be an alternative to naltrexone in alcoholism[48,49], but no studies in transplanted patients have been conducted so far.

Acamprosate, a NMDA-receptor antagonist used in alcoholism, has a very safe profile from a metabolic point of view, and it might be a good candidate for use in transplanted patients[32,33]. However, the existing studies have not been very optimistic in regarding the maintenance of abstinence[44].

Baclofen, a γ-aminobutyric acid receptor antagonist, was evaluated in a randomized, controlled study in patients with end-stage alcoholic liver disease, showing both safety and positive effects[50].

The human difficulty in addressing, accepting and sustaining change is widely known, above all in such a complex life situations as the transplantation process, and for this reason, psychotherapeutic treatment must be implemented during all stages of the transplantation process.

Understanding the patient means being attentive to greater difficulties or to factors that can trigger stress, which, in the case of an ALD patient, can lead to a lapse or a relapse, contributing to worse adherence, which can in turn translate into increases in morbidity and mortality. In this manner, it is important to develop coping strategies to face the factors that trigger stress and that could lead to a relapse. There are several structured models of psychotherapeutic intervention, the most common being MATCH (Matching Alcoholism Treatments to Client Heterogeneity), which has shown similar efficacy to 12-step treatment, cognitive

Table 1 Possible predictors of post-transplant relapse

Good prognosis
Pre-transplantation abstinence > 6 mo (controversial)
Social support
Abuse *vs* alcohol dependence
Good adherence in a broad sense
Poor prognosis
Family history of alcoholism
Non-acceptance of diagnosis related to alcohol use
Comorbidity with other substance abuse (controversial)
Psychiatric history (psychosis, personality disorder)
Quantity of alcohol/d
Number of years of use

behavioral therapy and motivational enhancement therapy[51].

CONCLUSION

Currently, ALD is one of the most common indications for liver transplantation, and post-transplantation survival and other predictors of the clinical course in ALD patients do not differ significantly from those of other types of transplanted patients, as long as there is no relapse of drinking.

However, relapse of drinking can have catastrophic consequences. Therefore, it is paramount to perform a psychosocial assessment of the candidate for transplantation and to identify relapse risk factors.

Table 1 lists the possible factors in relapse of drinking (poor and good prognostic factors), according to the literature.

This assessment allows not only for improvement of the selection of the patients who are to be transplanted but also for the design of psychopharmacological/psychotherapeutic interventions whenever necessary (in both the pretransplantation and post-transplantation periods), thus preventing and monitoring relapse.

This treatment should be integrated within the transplantation team and family (or significant others) and should be continued for at least one year post-transplantation[17].

Therefore, the authors suggest that ALD patients should now be considered a challenge rather than a threat - a challenge requiring special efforts by transplantation teams. Other types of transplanted patients, such as those with FAP (familial amyloid polyneuropathy), a rare genetic disease, for which the most accepted treatment is liver transplantation, can also represent an additional concern in terms of psychosocial adaptation to liver transplantation, and they should also receive special attention[52].

The authors also suggest that other factors from the immediate post-transplantation period that could also be important relapse risk factors or relapse-preventing factors could be investigated in this context. Factors such as post-transplantation coping strategies and mental status (*e.g.*, depression and anxiety levels) can predict several markers of good and poor medical and psychosocial outcomes, such as adherence to medication, quality of life, *etc.*[53], and could also be important factors in predicting alcohol relapse.

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TOPIC HIGHLIGHT

2015 Advances in Liver Transplantation

Posttransplant lymphoproliferative disorders following liver transplantation: Where are we now?

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Abstract

Liver transplantation has emerged as a life-saving treatment for several patients with acute liver failure, end stage liver disease and primary hepatic malignancies. However, long term immunosuppressive therapy aiming to reduce the risk of transplant rejection increases the incidence of several complications including malignancies. This is illustrated by the observation of a high ratio between observed and expected cases of lymphoproliferative disorders following liver transplantation. Despite a huge heterogeneity in morphological appearance of these disorders ranging from reactive-like lesions to real lymphomas, they are collectively termed posttransplant lymphoproliferative disorders. In this review we will provide an overview of this rare but challenging disorder as a complication of liver transplantation.

Key words: Epstein Barr virus; Liver transplantation; Posttransplant lymphoproliferative disorders

Core tip: Prevention of organ rejection following solid organ transplantation requires long term immunosuppressive therapy, leading to an increased risk of infections and malignancies. Posttransplant lymphoproliferative disorder (PTLD) comprises one of the most serious complications following transplantation with high morbidity and mortality rates. In this article we will review the different aspects on PTLD following liver transplantation.

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INTRODUCTION

Posttransplant lymphoproliferative disorder (PTLD) is serious complication of both solid organ (SOT)

and hematopoietic stem cell transplantation (HSCT). From a pathological point of view PTLD can vary from an infection-like appearance to a frank lymphoma. In about 70% of the cases Epstein Barr virus (EBV) is involved, whereas pathogenesis in the remaining cases is less clear. The most important risk factors for PTLD are EBV status at time of transplantation, type of transplanted organ and duration and type of immunosuppressive regimen. Reconstitution of the immune system, by reduction or withdrawal of immunosuppressive therapy, is considered the mainstay of therapy, although additional treatment is mandatory in a large proportion of patients. In this article we will review incidence, risk factors, diagnosis, treatment and prognosis of PTLD, focusing in particular on patients with liver transplantation.

INCIDENCE

Incidence data on PTLD in a transplant population may be underestimated given the lack of large prospective data, making retrospective single or rarely multicenter studies and large transplant registries the main information source. Population based cohort studies have shown that the standardized incidence ratio equals 10 for non-Hodgkin lymphoma and 3.5 for Hodgkin lymphoma following SOT[1].

However, the incidence of PTLD largely depends on the type of organ transplanted. Initially liver transplantation was associated with a relatively high risk for PTLD development compared to other transplanted organs[2]. However, in contrast to other solid organ transplantations, the risk seems to be decreasing due to a tendency to diminish and even discontinue all immunosuppressive therapy in a proportion of adult patients[3,4]. Similar, in pediatric liver transplant recipients, the incidence of PTLD has decreased due to preventive and especially preemptive modulation of immune suppressive therapy based on systematic EBV viral load monitoring[5,6].

RISK FACTORS

Several risk factors for development of PTLD have been described. The three most important are EBV mismatch, the type of transplanted organ and the use and duration of the immunosuppressive regimen.

EBV mismatch

Epidemiological studies in pediatric solid organ transplant recipients have shown that primary EBV infection from an EBV positive donor organ is the most important risk factor for development of PTLD, which was also confirmed in adult transplant populations. The major role of EBV in the development of PTLD is due to the dramatic decrease of EBV specific cytotoxic T lymphocytes caused by immune suppressive medication. This loss of immune surveillance may lead to uncontrolled proliferation of EBV-infected B cells. In a large Collaborative Transplant Study EBV negative serostatus at the time of transplantation was associated with a significant increased PTLD risk in kidney and heart transplant recipients. However, this was not the case following liver transplantation in which the risk was unaffected by the EBV serostatus[7]. This unexpected finding was challenged by a more recent analysis of a United States Scientific Registry of Transplant Recipients study, showing that recipient EBV seronegativity is significantly associated with risk for PTLD in heart, kidney but also liver transplantation with unadjusted hazard ratios (HR) of 6.528, 5.005 and 2.615 respectively. This lower HR in liver transplantation seems to be attributed to the higher baseline risk in EBV seropositive liver transplant patients[8]. The reason for this finding is not known, but may be related to the higher lymphoid mass of the transplanted liver, increasing the risk for EBV reactivation and subsequently development of PTLD[7].

Type of organ transplantation

The risk for PTLD development clearly varies according to the transplanted organ. Opelz *et al*[9] conducted a large retrospective study analyzing data from the Collaborative Transplant Study database. In this study the authors observed a 5 year relative risk (RR) for non-Hodgkin lymphoma of 29.9 following liver transplantation. RR was highest in lung-heart transplantation, followed by lung, heart, liver, pancreas and deceased donor kidney transplantation. This increased risk -for all types of transplantation- was most pronounced in the pediatric population, reflecting the higher percentage of EBV negative serostatus in children. In our own center we performed a retrospective analysis on 140 biopsy-proven PTLD cases collected during a 20-year period (1989-2010), confirming the organ-dependent differences in PTLD risk. Highest risk was observed in heart (5.0%), followed by lung (3.2%), liver (2.8%), hematopoietic stem cell (1.7%) and kidney (1.5%) transplant recipients, with an overall incidence in the whole transplant population of 2.12%. For statistical reasons heart-lung transplant patients were classified as lung transplant recipients, whereas no PTLD was seen following multivisceral transplantation, but this is probably due to the small (n = 9) number of this type of transplantation in our center during the studied period[10]. Other studies have shown incidence rates of 20% in both multivisceral and heart-lung transplantation. One of the largest series including 4000 consecutive liver transplant patients during the period 1981-1998 has been reported by the Pittsburgh group, who observed a PTLD incidence of 4.3% following liver transplantation, with clear difference between children (9.7%) and adults (2.9%)[11]. Possible reasons for the large differences in incidences between different organs include the fact that more

intensive immune suppressive therapy is required in high risk patients and that a larger burden of lymphoid tissue may increase the risk for EBV infection[12].

Immunosuppressive regimen

The often lifelong required intake of immunosuppressive medication is another important risk factor of PTLD development. Given the fact that most transplant protocols use combination regimens including induction and maintenance therapy, it is very difficult to determine the impact of each drug separately. However, although often controversial, some agents seems to be associated with development of PTLD, whereas others can even be considered protective.

Early studies have shown that the use of calcineurin inhibitors, both cyclosporine and tacrolimus, is associated with an increased risk for development of PTLD. Due to the stronger immunosuppressive properties of tacrolimus, this agent seems to be associated with a higher risk compared to cyclosporine in different organ types, including liver transplantation[13]. In contrast to the use of calcineurin inhibitors in liver transplantation the antimetabolite mycophenolate mofetil does not seem to increase the risk for PTLD, which is also observed in other organ transplantations[14]. Mammalian target of rapamycin (mTOR) inhibitors, often also referred to as proliferation signaling inhibitors, are very attractive agents given their combination of both immunosuppressive and antiproliferative characteristics. Currently two of these agents are used in organ transplantation, namely sirolimus and everolimus. In 2013 everolimus was approved in the United States and in Europe to prevent organ rejection in adult liver transplant patients. In a small study of 50 pediatric transplant patients, including 26 liver transplant recipients, the use of sirolimus combined with reduced dose tacrolimus was not associated with an increased risk for PTLD[15]. On the other hand, trials incorporating mTOR inhibitors in other organ transplantation have shown conflicting results with respect to the risk for PTLD development[16-18]. In liver transplantation, the use of combining everolimus with low dose tacrolimus may be a promising approach with acceptable tolerability, preserved renal function and decreased PTLD risk[19].

Most organ transplantation registry studies have shown a clear association between the use of polyclonal T cell depleting antibodies, in particular anti-thymocyte globulins, and the occurrence of PTLD[9]. Similar, the use of the monoclonal anti-CD3 antibody muromonab CD3 (= OKT3) was associated with an increased risk for PTLD development in a monocentric study including 1206 adult liver transplant recipients[20]. Given the depletion of both B- and T-cells when using the anti-CD52 monoclonal antibody alemtuzumab, this agents offers the theoretical advantage of protection against B cell proliferation. However, no clear data confirming this hypothesis do exist in liver transplantation[21]. Recently selective depletion of activated T cells with anti-interleukin-2 receptor (CD25) monoclonal antibodies (basiliximab and daclizumab) have been used extensively as induction therapy in liver transplantation, without increasing the incidence of PTLD[9,22,23].

In a recently published Cochrane systematic review all different types of polyclonal and monoclonal depleting and non-depleting antibodies used as induction therapy in liver transplantation were evaluated in order to assess their benefits and disadvantages. In this analysis 19 randomized clinical trials with a total of 2067 liver transplant recipients were included. No specific harm in general (PTLD in particular) was found when comparing each antibody with no induction therapy. However the authors concluded that more well designed clinical trials are needed because of the high risk of bias in the studied trials, the small numbers of randomized trials and the limited numbers of participants and examined outcomes in these trials[24].

Other risk factors

Many other risk factors for development of PTLD in general have been described and proposed, although their relationship remains controversial. In liver transplant patients the underlying disorder and non-EBV viruses also have been proposed as risk factors for development of PTLD.

In a German monocentric retrospective analysis the authors observed a significant relation between pretransplant steroid treatment due to immunological disorders and liver transplantation for autoimmune hepatitis and the occurrence of PTLD[25].

About one third of the PTLD cases is not EBV-associated[12]. In these cases other infectious agents may be involved or the malignant cells may have lost EBV expression[26]. Different viruses have been proposed as important contributors in the pathogenesis of PTLD, but as will be discussed in the next paragraph, no conclusions can be made on their exact role.

Hézode *et al*[27] reported on an increased risk for PTLD development in liver transplant patients with underlying hepatitis C cirrhosis. However, a large cohort study in SOT recipients failed to confirm this observation[28]. This apparent lack of association between hepatitis C and development of PTLD clearly contrasts to its role in lymphomagenesis in immune competent patients. Recently a large population-based Swedish study including 135 PTLD cases following solid organ transplantation suggested hepatitis C virus to be associated with late onset PTLD, which also needs confirmation in larger studies[29]. Although less well studied, Zhang *et al*[30] observed an increased incidence of PTLD in liver transplant recipients transplanted

Table 1 World Health Organization classification posttransplant lymphoproliferative disorder

	Early lesions	Polymorphic PTLD	Monomorphic PTLD
Underlying architecture	(Partially) preserved	Destructed	Destructed
Cells	Plasma cells, small lymphocytes and immunoblasts	Complete spectrum of B cell maturation	Fulfill criteria for lymphoma
Immunohistochemistry	No diagnostic value	Mixture of B and T cells	Most cases CD20 positive
EBV	100%	> 90%	+/- 70%
Clonality	In most cases polyclonal	Variable	Monoclonal
Oncogenic mutations	No	Variable (BCL6)	Oncogenes (N-Ras, c-MYC,…) and tumor suppressor genes (p53,…)

PTLD: Posttransplant lymphoproliferative disorder; EBV: Epstein Barr virus.

for benign liver diseases with hepatitis B virus (HBV) compared to HBV-negative patients. Available data on the impact of Cytomegalovirus (CMV) both in liver and other organ transplantations are very controversial, so currently no conclusions can be drawn regarding the role of CMV in PTLD development[31-33].

In summary we can conclude that EBV mismatch, type of transplanted organ and immunosuppressive regimens are major determinant factors in the risk for PTLD development following solid organ (and liver) transplantation. The impact of other factors, including underlying disorder and non-EBV viruses remains controversial.

CLINICAL PRESENTATION

The clinical presentation of patients with PTLD is very heterogeneous. Whereas some patients have no symptoms or a mononucleosis-like presentation, other present with very aggressive disease including rapid evolution to multi-organ failure. Large mono- and multicentric case series following solid organ transplantation reveal a high incidence of extranodal invasion (62%-79%), including bone marrow (15%-17%), gastrointestinal tract (23%-56%) and central nervous system involvement (5%-13%). The majority of patients present with advanced disease (Ann Arbor stage Ⅲ-Ⅳ in 66%-72%). In accordance with the increased survival of patients following organ transplantation, most recent series show that the majority of PTLD cases are late onset cases, developing more than one year following transplantation (61%-72%), with up to 21% occurring more than 10 years post transplantation[10,11,29,34]. A minority of cases are characterized by early onset (first six months) presentation and are often limited to the allograft[35].

DIAGNOSIS

Once diagnosis of PTLD is suspected prompt diagnostic investigations are essential in order to confirm or exclude the diagnosis and to initiate treatment as soon as possible. Although diagnosis can be assumed based on clinical presentation and EBV monitoring in peripheral blood, the gold standard for diagnosis remains biopsy with histopathological and immunohistochemical examination. Based on morphological and immunohistochemical findings and on the structure of the underlying lymph node/organ, the World Health Organization distinguishes four major categories of PTLD (Table 1)[36]: (1) early lesions (plasmacytic hyperplasia and infectious mononucleosis-like lesions); (2) polymorphic PTLD; (3) monomorphic PTLD; and (4) hodgkin lymphoma/Hodgkin-like lymphoma.

STAGING

Adequate staging examinations are needed aiming to define the extent of the disorder. Staging tools include: CT scan abdomen/thorax/pelvis, bone marrow examination and in case of suspicion of central nervous system invasion magnetic resonance imaging of the brain and/or analysis of cerebrospinal fluid. Based on these findings all cases can be categorized in stages according to the Ann Arbor classification, classifying patients based on the number of involved lymph node regions, the localization of nodal involvement and the presence of organ invasion. Stage Ⅰ and Ⅱ are considered limited disease, whereas stage Ⅲ and Ⅳ point to a more advanced or disseminated disease (Table 2)[37].

The high frequency of extranodal involvement in PTLD and the relative contra-indication for the use of intravenous contrast in patients with compromised calcineurin inhibitor-induced renal dysfunction have led to a particular interest in the use of ^{18}fluorodexyglucose- positron emission tomography (FDG-PET) scan in diagnosis and staging of PTLD. We evaluated the use of FDG-PET in 170 cases with suspected or biopsy-confirmed PTLD following solid organ or hematopoietic stem cell transplantation, confirming its high sensitivity and specificity and showing an excellent ability to differentiate PTLD from non-malignant disorders. Potential pitfalls include central nervous system involvement and -isolated-allograft localization in heart and kidney transplant recipients, for which PET scan is not the ideal imaging modality[38]. Similar results were observed in two

Table 2 Ann Arbor staging system for lymphoproliferative disorders

Stage Ⅰ	Involvement of a single lymph node region (Ⅰ) or one extralymphatic site (IE)
Stage Ⅱ	Involvement of two or more lymph node regions, at the same side of the diaphragm (Ⅱ) or local extralymphatic extension plus one or more lymph node regions at the same side of the diaphragm (IIE)
Stage Ⅲ	Involvement of lymph node regions on both sides of diaphragm (Ⅲ) which may include the spleen (IIIS) or accompanied by local extralymphatic extension (IIIE) or both (IIIES)
Stage Ⅳ	Diffuse or disseminated involvement of one or more extralymphatic organs or sites, with or without associated lymphatic involvement

Each stage number is followed by either A (absence of B-symptoms) or B (presence of B-symptoms: unexplained weight loss > 10% baseline during 6 mo before, unexplained fever > 38 ℃, night sweats).

other studies in which the authors also compared PET findings with those obtained with more conventional imaging modalities[39,40].

PREVENTION

Improved knowledge on the important contribution of EBV in the pathogenesis of PTLD and ongoing concerns regarding poor prognosis of the disorder with significant morbidity and mortality, has moved the attention to prevention of the disorder.

Prophylactic therapy

The use of antiviral agents, especially the nucleoside analogues acyclovir and ganciclovir, in prophylaxis and treatment was already explored more than thirty years ago, with limited benefit[41]. Information on the effect of prophylactic use of viral agents with regard to the development of PTLD is limited. In a randomized controlled trial in 48 pediatric liver transplant recipients prophylactic treatment with two weeks of intravenous ganciclovir alone (10 mg/kg per day) was compared to two weeks of ganciclovir followed by 50 wk of high-dose oral acyclovir (4 × 800 mg/m² per day). Patients who were treated with prolonged use of acyclovir did not show an increased frequency of PTLD in this study[42]. In a recent multicenter case-control study Funch *et al*[43] examined the impact of acyclovir and ganciclovir on the development of PTLD following kidney transplantation. This analysis showed that prophylactic anti-viral therapy, especially when using ganciclovir, provides a significant protection against early onset (< 1 year following transplantation) EBV-driven PTLD. However, these findings were not confirmed in a large retrospective registry study including 44.828 deceased-donor kidney transplant recipients, showing that prophylactic treatment with antiviral drugs did not reduce the risk of PTLD[44].

The use of intravenous immune globulins (IVIG) might be another promising therapy in PTLD. However, efficacy of this approach is not very clear as often similar therapies are given[45]. As the results of two trials (one in kidney and one in pediatric liver transplant recipients) examining the effect of anti-CMV IVIG showed controversial, the use of IVIG early in transplant programs remains questionable[44,46].

Preemptive therapy

With the availability of quantitative polymerase chain reaction, monitoring of EBV viral load has become common practice in many centers taking care of transplant patients. Potential preemptive strategies based on EBV viral load monitoring include reduction of immune suppressive medication, antiviral medication and/or administration of rituximab, a monoclonal anti-CD20 antibody. McDiarmid *et al*[47] reported on their experience using a protocol incorporating serial peripheral blood EBV viral load monitoring following pediatric liver transplantation. In patients with increasing viral copy number, tacrolimus was decreased and ganciclovir was re-initiated or continued. In a similar single center study Lee *et al*[5] proposed a similar approach with reduction of immune suppression in case of high EBV load in 43 pediatric liver transplant patients and compared them with a historical control group. In both studies the authors concluded a significant decrease in PTLD incidence was observed with the introduction of this preemptive strategy.

TREATMENT

Given the rarity of the disorder and due to the lack of randomized phase Ⅲ trials, optimal treatment of PTLD is currently not clearly defined. This is illustrated by the recently published guidelines from the British Committee for Standards in Haematology and the British Transplantation Society, showing low levels of evidence and weak recommendations grades for the different therapeutic options[48].

The development of PTLD always implies a high degree of overimmunosuppression. This observation explains why reduction of immunosuppression is the main therapeutic intervention which should be initiated promptly, leading to restoration of the EBV-specific T cell response.

Restoration of the immune system

Reduction of immunosuppression: As soon as the diagnosis of PTLD is made, prompt initiation of RIS is recommended. In most cases antimetabolites are discontinued, calcineurin inhibitor dose is reduced with 50% and steroids or continued[48,49]. If the clinical situation of the patients allows, the effect should be re-evaluated after two to four weeks. Response rates to RIS alone in PTLD have a very wide variation, reflecting the lack of standardization

with respect to duration of RIS before re-evaluation, response criteria and reduction regimen. The impact of RIS on PTLD following liver transplantation is difficult to assess as most large series contain cases following different kinds of organ transplantation. In a large monocentric analysis from the University of Pennsylvania, including 67 SOT recipients (16 liver transplant patients) with PTLD, RIS alone was associated with an overall response rate of 45% and a complete response rate of 37%. The most important factors predictive for response to RIS alone were the absence of bulky disease (> 7 cm), early stage (Ann Arbor Ⅰ-Ⅱ) and lower age (< 50 years)[50,51]. In a large Swedish study 135 PTLD cases following solid organ transplantation (SOT) were analyzed, including 19 (14%) liver transplant recipients. Twenty-one patients were treated with RIS alone, of which 57% had a complete remission (CR)[29]. However, in a prospective trial from Baltimore including 16 SOT recipients, only 6% responded to RIS alone with no CR, but no liver transplant recipients were included[52]. In a small retrospective analysis focusing on liver transplant recipients (n = 17) RIS alone was associated with a CR rate of 46%[53].

In conclusion, RIS should be initiated in all patients presenting with PTLD following liver transplantation. If the condition of the patient doesn't require urgent additional therapy, a re-evaluation should be performed after 2 to 4 wk. During RIS, regular monitoring of transplant function is essential, as RIS is associated with an increased risk of organ rejection.

As already discussed before mTOR inhibitors may be a promising approach in the treatment of malignancies in transplant recipients, given their immunosuppressive and antiproliferative capacities. Recently, Ashrafi *et al*[54] published their experience with 13 kidney transplant recipients who were treated with everolimus following diagnosis of PTLD, indicating promising results regarding both disease control and graft survival. This may be in particular a very attractive approach in liver transplant patients, given the beneficial effect of everolimus in prevention of transplant rejection[19].

Adoptive immunotherapy: The use of EBV specific cytotoxic lymphocytes has shown impressive results in refractory PTLD cases with a very good toxicity profile, as reviewed by Merlo *et al*[55]. However, we will not discuss this therapy in detail as wide applicability has been limited so far.

Anti- B cell therapy

Surgery and radiotherapy: Surgery and radiotherapy should only be used in localized disease, especially in early lesion PTLD[48,50]. Other indications for radiotherapy include palliative symptom control and treatment of isolated central nervous system-PTLD[56].

Chemotherapy: Although chemotherapy (mostly CHOP) was initially considered standard therapy, especially after failure of RIS, treatment related mortality seemed to be very high compared to immune competent patients[57-59]. However, as will be discussed in the next part, the use of rituximab has substantially changed the treatment of patients presenting with CD20-positive B-cell PTLD, making omission of chemotherapy possible in a substantial proportion of patients. However, in case of aggressive CD20 negative PTLDs, upfront chemotherapy is mandatory in most cases[48].

Monoclonal anti-B cell therapy: Several prospective phase Ⅱ trials have assessed the role of rituximab, a chimeric monoclonal anti-CD20 antibody, in PTLD. Based on the results of these trials, showing overall response rates ranging between 44% and 64% combined with a favorable toxicity profile, rituximab has emerged as standard therapy for CD20-positive PTLD with inadequate response to RIS[60-64].

Recently Trappe *et al*[65] reported on the results of the large prospective phase Ⅱ PTLD-1 trial examining the sequential use of rituximab and CHOP chemotherapy in 70 patients presenting with CD20-positive PTLD following SOT, including liver transplantation. This trial demonstrated the efficacy (90% ORR with 67% CRR) and safety of sequential treatment. As the response to rituximab predicted overall survival, the trial was amended in 2007 introducing risk stratification (risk stratified sequential treatment) according to the response to rituximab. The final analysis of this approach needs to be awaited before final conclusions can be made.

Anti- EBV therapy

Antiviral therapy: The use of antiviral treatment has not been assessed in prospective trials. In addition, as already mentioned before, nucleoside analogues don't seem to be efficient as most EBV positive tumors do not express viral TK.

Arginine butyrate: Recently very promising results have been described with the short-chain fatty acid arginine butyrate, a selective activator of viral TK making the tumor sensitive to treatment with nucleoside analogues. Combining arginine butyrate with ganciclovir in the treatment of 6 refractory PTLDs was feasible and showed an impressive response rate of 83%[66].

PROGNOSIS

In general the prognosis of PTLD following SOT is poor with 3-year and 5-year overall survival of approximately 50%-60% and 40% respectively[10,29,34], although sequential therapy with rituximab and CHOP

chemotherapy shows improved overall survival (61% at 3 year)[65]. Kremers *et al*[20] observed a 5-year OS of 40.8% in 37 patients with PTLD following liver transplantation. Importantly, a significant percentage of deaths (42% in our retrospective analysis) are not PTLD-related, but are due to other causes, in particular infections[10].

In our opinion and experience the International Prognostic Index score[67] -a risk score initially defined for immune competent patients with aggressive non-Hodgkin lymphoma, based on 5 independent risk factors: age > 60 year, elevated LDH, poor performance state, advanced Ann Arbor stage and presence of extranodal localizations- is also a reliable and predictive factor in patients presenting with PTLD, which was also confirmed in the PTLD-1 trial[10,68,69] An additional risk factor for poor prognosis in the PTLD-1 trial was the presence of a thoracic organ transplantation not responding to rituximab monotherapy[69].

CONCLUSION

Posttransplantation lymphoproliferative disorders remain an important cause of morbidity and mortality following solid organ transplantation in general and liver transplantation in particular. Although the overall PTLD incidence has increased during the last years, liver transplantation seems to be an exception to this general rule, probably due the tendency to diminish and even discontinue all immunosuppressive therapy in a proportion of adult patients and to the use of preemptive strategies, especially in the pediatric setting. Classical risk factors for PTLD include the EBV serostatus of the patient, the organ transplanted and the immunosuppressive regimen. Once PTLD is suspected, diagnostic evaluation and staging should be done as soon as possible, as pathological identification of the subtype and evaluation of the involved nodes and organs are critical factor for optimal treatment and prognostic stratification. As soon as the diagnosis is made, treatment should be initiated promptly by reducing immune suppressive therapy. In most cases this will be followed by systemic treatment with rituximab and/or chemotherapy.

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TOPIC HIGHLIGHT

2015 Advances in Alcoholic fatty liver disease

Non-invasive assessment of liver fibrosis in patients with alcoholic liver disease

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Abstract

Alcoholic liver disease (ALD) consists of a broad spectrum of disorders, ranging from simple steatosis to alcoholic steatohepatitis and cirrhosis. Fatty liver develops in more than 90% of heavy drinkers, however only 30%-35% of them develop more advanced forms of ALD. Therefore, even if the current "gold standard" for the assessment of the stage of alcohol-related liver injury is histology, liver biopsy is not reasonable in all patients who present with ALD. Currently, although several non-invasive fibrosis markers have been suggested as alternatives to liver biopsy in patients with ALD, none has been sufficiently validated. As described in other liver disease, the diagnostic accuracy of such tests in ALD is acceptable for the diagnosis of significant fibrosis or cirrhosis but not for lesser fibrosis stages. Existing data suggest that the use of non-invasive tests could be tailored to first tier screening of patients at risk, in order to diagnose early patients with progressive liver disease and offer targeted interventions for the prevention of decompensation. We review these tests and critically appraise the existing evidence.

Key words: Transient elastography; Cirrhosis; Prognosis; Histology; Collagen proportionate area; Fibrotest; AST-to-platelet ratio index; Diagnostic accuracy; Cost-effectiveness; Serum fibrosis markers

Core tip: Although there has been an explosive development and validation of non-invasive fibrosis tests particularly in viral hepatitis, data on patients with alcoholic liver disease are still scarce. We review these tests and critically appraise the existing literature. Evidence suggests that such tests could be tailored to first tier screening of patients at risk, in order to diagnose early patients with progressive liver disease and offer targeted interventions for the prevention of decompensation.

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INTRODUCTION

Chronic liver disease is one of the main causes of morbidity and mortality worldwide, as its most relevant complications, namely cirrhosis and liver cancer, account for approximately 2% and 1.4% of all deaths, respectively[1,2]. Excessive alcohol consumption is a major risk factor for chronic liver disease in industrialized countries, and is the predominant cause of liver disease in 48% cases of cirrhosis in the United States[3].

Alcoholic liver disease (ALD) is defined by anamnestic history of daily alcohol intake of at least 30 g and 20 g for men and women respectively, associated with evidence of liver injury[1].

ALD consists of a broad spectrum of disorders, ranging from simple fatty liver to more severe forms, namely alcoholic steatohepatitis (ASH) and cirrhosis, leading to life-threatening complications such as hepatocellular carcinoma (HCC) and liver failure. Fatty liver develops in more than 90% of heavy drinkers, however only 30%-35% of them develop more advanced forms of ALD. This suggests the role of other contributing factors, such as female sex, obesity, drinking patterns, dietary factors, cigarette smoking and non-sex-linked genetic factors. In particular, genetic factors such as the polymorphism of the patatin-like phospholipase domain-containing protein 3 are currently the focus of further research[3].

Early detection of cirrhosis is important in patients with ALD, as abstinence can prevent the advent of complications and improve prognosis[4,5]. In a cohort of 466 patients with ALD cirrhosis, 1-year mortality was 17% in the absence of baseline complications, progressively increasing to 20%, 29% and 64% in the presence of variceal bleeding, ascites, and encephalopathy, respectively[6]. Abstinence improves survival in patients with established ALD; in a study including 283 patients with ALD cirrhosis and a 5-year follow-up, a significant difference in survival between abstainers and drinkers was demonstrated, with corresponding rates of 63% and 45% respectively[7]. These data were confirmed by Verrill *et al*[8] in a 7-year follow-up of 100 patients with alcoholic cirrhosis.

The current "gold standard" for the assessment of alcohol-related liver injury is histology, obtained through liver biopsy. The histological examination provides information about liver architecture, presence and extent of steatosis, necroinflammation and fibrosis. Nevertheless, it is an invasive procedure with some limitations. Firstly, it is subject to sampling errors and intra and inter-observer interpretation variability, mainly due to the small portion of liver examined. Secondly, it is associated with patient discomfort and a small but significant risk of severe complications, such as hemobilia or bleeding[9].

Therefore, although liver biopsy remains essential in selected cases, there has been a growing interest in non-invasive methods for the assessment of liver fibrosis.

LIVER FIBROSIS AND STAGING ASSESSMENT

The fibrogenic process is a maladaptive wound-healing response to a generic liver injury characterized by excessive production and accumulation of collagen and other extracellular matrix proteins by activated hepatic stellate cells and portal fibroblasts[3,10]. When an imbalance between extracellular matrix production and degradation occurs, fibrosis progresses[11].

In ALD, fibrosis begins in the perivenular regions and extends to portal tracts, leading to the formation of central-portal or portal-portal bridging fibrosis. If the alcoholic injury persists, fibrosis and hepatocyte regeneration result in nodule formation and finally in cirrhosis[10].

Histological examination estimates fibrosis by using a semi-quantitative "staging" scoring system that takes into account both fibrosis and architectural changes[12,13]. Currently different histological semi-quantitative scoring system are available, such as the Ishak and METAVIR scores for viral hepatitis or the Brunt and Kleiner score for non-alcoholic fatty liver disease. These classification systems use numerical categorical labels to describe histological features such as steatosis or necroinflammation. One of their major limitations is the assignment of fixed numerical scores to continuous histological variables, so that the numbers provide more a descriptive feature rather than an actual measurement. Nevertheless, there is no direct correlation of these scoring labels with the amount of fibrosis[12]. This limitation is reflected in the development and evaluation of non-invasive fibrosis markers, where the continuous value provided by the non-invasive fibrosis test is used for the diagnosis of the semi-quantitative stage that describes both architecture and distribution of fibrosis, and thus probably results in a higher number of misclassifications (Table 1).

Recently, a quantitative method of measuring fibrous tissue, through digital image analysis of the proportion of collagen in liver tissue, namely collagen proportionate area (CPA), has been developed[12-14]. CPA is a direct measure of the amount of fibrosis in the liver and could be better used for the validation of non-invasive fibrosis markers but also for the evaluation of future anti-fibrotic treatment.

Table 1 Non-invasive serum tests for the assessment of liver fibrosis and corresponding stages of alcoholic liver disease diagnosed

Test	Variables	ALD stage assessed
AST:ALT ratio	ALT, AST	≥ F1, F4
HA	HA	F4
PGA	PT, GGT, apolipoprotein-A1	F4
PGAA	PT, GGT, apolipoprotein-A1, α2-macroglobulin	
FibroTest®	Haptoglobin, α2-macroglobulin, apolipoprotein-A1, bilirubin, GGT	≥ F3, F4
FibrometerA®	Age, weight, glucose, AST, ALT, PLT count, ferritin	≥ F3, F4
FIB-4	Age, AST, ALT, platelet count	≥ F3
Hepascore®	Age, sex, bilirubin, GGT, α2-macroglobulin, HA	≥ F3, F4
APRI	AST, platelet count	≥ F2, F4
ELF test™	HA, PIIINP, TIMP-1	≥ F3, F4
Forns index	Age, platelet count, total cholesterol, GGT	≥ F3, F4

ALD: Alcoholic liver disease; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; PT: Prothrombin time; GGT: γ-glutamyl transpeptidase; PLT: Platelets; PIIINP: Terminal peptide of procollagen Ⅲ; TIMP-1: Tissue inhibitor of metalloproteinase 1; HA: Hyaluronic acid.

NON-INVASIVE FIBROSIS TESTS

A diversified set of both serum and imaging potential markers has been developed for the non-invasive assessment of liver fibrosis. Serum biomarkers are classed as indirect (class Ⅱ) or direct (class Ⅰ). The development of advanced imaging technique has led to the availability of additional tools to stage liver fibrosis. The advantages of these new methods are the widespread availability, the non-invasiveness and the high reproducibility. Therefore, when validated as sufficiently accurate, they represent a perfect tool for risk stratification, staging fibrosis and long-term follow-up of patients.

Serum biomarkers

Class Ⅱ serum biomarkers or indirect fibrosis tests: Class Ⅱ biomarkers consist of routinely performed serological tests, which evaluate common altered liver parameters, such as transaminases, platelet count or albumin. They are not surrogate markers of matrix turnover or the fibrogenic process in the liver, but rather reflect hepatic function or inflammation. They are usually combined into score systems or panels where other demographic features are included, such as presence of diabetes or age, in order to better classify fibrosis stages[15-17]. These panels have a high and a low cut-off for the diagnosis of a specific fibrosis stage, in order to minimize the number of false positive and false negative respectively. Therefore, a number of patients fall in the "indeterminate zone" between the two thresholds, so that additional investigations to classify such patients are required.

Class Ⅰ serum biomarkers or direct fibrosis tests: Class Ⅰ biomarkers reflect the products derived from the turnover of the extracellular matrix during the fibrogenic process. During this process, there is a consistent increase in the serum levels of fibrogenic cytokines (*e.g.*, tumour-growth factor β), extracellular matrix components [*e.g.*, hyaluronic acid (HA)], degradation products (*e.g.*, procollagen Ⅳ C peptide), and enzymes involved in these processes (*e.g.*, tissue inhibitor of metalloproteinases TIMP-1)[15]. Such biochemical tests are currently performed in designated laboratories and are usually part of complex panels.

IMAGING TECHNIQUES

Ultrasound, CT and MRI can only detect the presence of hepatic steatosis or signs of cirrhosis and portal hypertension, with little contribution to the identification of patients with less advanced stages of fibrosis. Therefore, in recent years new imaging techniques have been developed in order to overcome this limit and emerging data are accumulating, particularly in viral hepatitis. The architectural changes in the liver driven by inflammation and deposition of fibrotic tissue lead to alterations in the microstructure reflected by an increase in the liver stiffness. This can be measured by using elastography principles, which are based on the propagation of a mechanical share wave through the liver parenchyma; the propagation velocity reflects the liver stiffness. Moreover, MRI technique also adapted to assess hepatic fibrosis by modifying phase-contrast imaging sequence to detect the shear waves within the liver.

Transient elastography (TE) or FibroScan® (Echosens; Paris, France) was the first imaging modality used to detect liver fibrosis. It is an ultrasound-based technique that uses an ultrasonic transducer probe (5 MHz), which emits low-frequency vibrations into the liver, creating a propagating shear wave. The latter is detected by a pulse-echo acquisition, which then calculates its velocity. The results are expressed in Kilopascals (kPa) and the final value is the mean of ten valid measurements. In order to ensure a reliable determination of liver stiffness, an interquartile range for measurements within 30% and ratio of success rate of measurements > 60% are required[18]. Liver stiffness is measured in a volume of approximately 1 cm wide and 4 cm long, corresponding to nearly 1/500 of the whole liver volume, thereby representing a sample 100 times greater than the one obtained from a liver biopsy.

The results range from 2.5 to 75 kPa, however a validation of exact stiffness cut-offs for specific fibrosis stages is still lacking[19]. Liver stiffness cut-offs for the diagnosis of extensive fibrosis or cirrhosis could be different according to the cause of the underlying

liver disease, possibly because liver stiffness mainly reflects the amount of liver fibrosis without taking into consideration its distribution within the liver, which the fibrosis staging systems are based on[20].

Fibroscan® has important limitations that need to be taken into account. Firstly, it is technically difficult in patients with visceral obesity, elevated BMI or narrow rib interspaces. Secondly, stiffness values are artificially increased in the presence of congestive heart failure, acute hepatitis, infiltrative liver disease like amyloidosis or if the measurement is performed post-prandially. In a 5-year prospective study by Castéra *et al*[21], which included 13369 patients, the probability of failure and/or unreliable results of Fibroscan® was 18%; this failure was independently associated with obesity, in particular increased waist circumference, and limited operator experience (< 500 examinations performed). The rate of unreliable results is not taken into account when the accuracy of TE is reported in studies, thereby resulting in an overestimation of its performance. A new XL probe for obese patients results in different stiffness cut-offs than the M probe and is still undergoing validation.

Acoustic radio force impulse (ARFI) evaluates the elastic properties of a hepatic region of interest while performing a real-time B-mode conventional hepatic ultrasonography, so that large blood vessels or ribs could be avoided. The elastography system is directly integrated on a standard ultrasonography device (Acuson 2000/3000 Virtual Touch™ Tissue Quantification, Siemens Healthcare, Erlangen, Germany) and short acoustic high-intensity impulses with a fixed frequency of 2.67 MHz are sent into the tissue inducing a tissue displacement and the propagation of shear waves away from the region of excitation. The propagation velocity of these shear waves is expressed in m/s and correlates to the tissue stiffness. The tissue displacements are inversely proportional to the stiffness of the tissue, so that a stiffer region of tissue exhibits smaller displacements than a more compliant region[22]. A high correlation between ARFI elastography and Fibroscan® in staging of liver fibrosis has been demonstrated[23]. ARFI performance seems not to be affected by the presence of hepatic steatosis, whereas the influence of the inflammatory activity in the liver has been confirmed[24].

Supersonic Shear Imaging (SSI), also named ShearWave™ elastography, is a new technique based on the measurement of the velocity of a local shear wave through soft tissues. It uses an ultrasound transducer (Aixplorer, Supersonic Imagine, Aix-en-Provence, France), which emits a series of pulse waves at increasing depths, using a very wide frequency band ranging from 60 to 600 Hz. By generating a real-time colour mapping of the elasticity of the tissue explored coupled with a B-mode image, SSI provides a quantitative imaging of the tissue elasticity. The final value is the average the measurement obtained by selecting the region of interest by using both B-mode and SWE images[25,26]. Unlike Fibroscan, there are no established quality criteria for measurements using ARFI or SSI.

Magnetic resonance elastography (MRE) combines images produced by a traditional magnetic resonance (MR) system with a modified phase contrast technique able to depict the propagation of acoustic shear waves generated by a pneumatic driver. Elasticity values are expressed in KPa and are obtained as mean values measured in a region of interest within the liver[27]. This technique is still undergoing validation and is not used in routine clinical practice.

NON-INVASIVE ASSESSEMENT OF LIVER FIBROSIS IN ALD

Even if data are accumulating on the use of non-invasive tests for the assessment of liver fibrosis in ALD, nevertheless a small number of studies is currently available on such patients compared to other causes of liver disease. Therefore, although non-invasive fibrosis tests have greatly reduced the need for liver biopsy, particularly in patients with HCV, the evidence is still scant in ALD (Table 2).

Serum markers

Aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio includes AST and ALT. Values > 1, and especially > 2, are highly suggestive of an alcoholic aetiology of liver disease or the presence of cirrhosis[28,29].

The first serum panel used and validated for the detection of cirrhosis among drinkers is the PGA index, which consists of prothrombin index (PT), gamma glutamyl transferase (GGT) and Apolipoprotein A1[30]. Subsequently α2 macroglobulin has been added to form the PGAA index. PGAA was tested in a cohort of 525 alcoholic patients with different histological stages of fibrosis and performed better than PGA in detecting significant fibrosis or cirrhosis (correct classification in 70% of PGAA and 65% of PGA, $P < 0.001$). The derived PGAA cut-offs for excluding or diagnosing cirrhosis were $\leqslant 3$ and $\geqslant 9$ respectively. Moreover, in a sub-analysis of asymptomatic patients ($n = 316$), a PGAA cut-off of 7 had a sensitivity of 89% and specificity of 79% for the diagnosis of cirrhosis, suggesting a potentially important role in detecting early cirrhosis among drinkers[31].

APRI (AST-To-platelet ratio index) includes AST and platelet count as variables. It is calculated as (AST/upper limit of normal range)/platelet count (10^9/L) X 100. Wai *et al*[32] developed this index in a cohort of 270 HCV mono-infected patients and showed than an APRI cut-off of < 1.0 excluded cirrhosis with a negative predictive value of 98%, whereas a score > 2 was predictive of cirrhosis with a positive predictive value of 93%. On the other hand, cut-offs of < 0.5

Table 2 Non-invasive serum tests to diagnose fibrosis in patients with alcoholic liver disease and corresponding diagnostic indexes

Test	Cut-offs	F3			F4		
		Sensitivity	Specificity	AUROC	Sensitivity	Specificity	AUROC
PGA	< 2	NA	NA	0.84	0%	83%	NA
	> 9	NA	NA	NA	86%	100%	0.89
PGAA	< 3	NA	NA	0.86	NA	NA	NA
	> 12	NA	NA	NA	NA	NA	0.83
Hyaluronic acid, μL	> 55	83.0%	69%	NA	NA	NA	NA
	> 60	NA	NA	NA	100%	60%-86%[2]	NA
	> 100	NA	NA	NA	89%	87%	NA
	> 250	NA	NA	NA	100%	69%	0.78
APRI	< 0.5	NA	NA	NA	NA	NA	NA
	> 1.5	13.2%	77.6%	0.43-0.7[2]	NA	NA	NA
	< 1	NA	NA	NA	NA	NA	NA
	> 2	9.4%	96.6%	NA	16.9%	86.4%	0.56-0.79[2]
FIB-4	< 1.45	NA	NA	0.7	NA	NA	0.8
	> 3.25	NA	NA	0.7	NA	NA	0.8
FibroTest®	< 0.3 (0.3-1.58[1])	84.0%	66%	0.79	100%	50%	0.84
	> 0.7 (0.7-1.0[1])	55.0%	93%	0.83	91%	87%	0.94
FibroMeter®	NA	91.8%	92.3%	0.82-0.88[2]	91.8%	92.3%	0.85-0.94[2]
ELF test™	< 0.431	93.3%	100%	0.94	93.3%	100%	0.94
	> 9.5	74.4%	92.4%	NA	74.4%	92.4%	NA
Hepascore®	NA	NA	NA	0.76-0.83[2]	NA	NA	0.76-0.92[2]
Forns	NA	NA	NA	0.38	NA	NA	0.38

[1]A single cut-off has not been validated; [2]A single value has not been proposed. NA: Not available; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; AUROC: Area under receiver operator characteristic curve; GGT: γ-glutamyl transpeptidase; PLT: Platelets; PT: Prothrombin time; PIIINP: Terminal peptide of procollagen Ⅲ; TIMP-1: Tissue inhibitor of metalloproteinase 1; APRI: Aspartate aminotransferase to platelets ratio; ELF: Enhanced liver fibrosis.

and > 1.5 excluded and confirmed the presence of significant fibrosis (METAVIR F2), respectively. Conversely, poorer results have been found in patients with ALD, probably due to the direct effect of alcohol on platelet count and AST[33]. In a cohort of 507 patients with ALD, APRI values of > 1.5 had sensitivity and specificity of 13.2% and 77.6% respectively for the detection of significant fibrosis, and 16.9% and 86.4% respectively for the diagnosis of cirrhosis at a cut-off of 2, thus suggesting a limited utility in clinical practice[33]. The relatively poor diagnostic performance of APRI was confirmed in a comparative study of TE and non-invasive serum tests in a cohort of 103 alcoholic patients. APRI yielded the lowest AUROC at 0.56, while FibroTest and PGAA had AUROCs of 0.84 and 0.83 respectively[34].

The Forns index is based on four routine clinical variables: age, platelet count, cholesterol levels and GGT. It has been developed to predict advanced fibrosis in a cohort of 476 chronic C hepatitis patients[35]. Both APRI and Forns scores showed low performance in detecting both advanced fibrosis and cirrhosis in a cohort of 214 patients with ALD, with AUROCs of 0.38 and 0.59 for the former and 0.67 and 0.38 for the latter, respectively[36]. Similarly, in a cohort of 49 patients with ALD, TE was significantly better for diagnosing advanced fibrosis than APRI and Forns, with corresponding AUROCS of 0.766, 0.611 and 0.648[37].

The performance of single direct serum markers has also been studied in patients with ALD. Of them, more data is available on HA. In a systematic review by Parkes *et al*[38], there were 15 identified studies on HA in ALD, of which only 7 reported cut-offs for the identification of severe fibrosis or cirrhosis. Nevertheless, the number of participants was small (range *n* = 70-247) and varying cut-offs for the diagnosis of cirrhosis were provided (60-300 μg/L). Sensitivity and specificity ranged from 87%-100% and 60%-89%, while only 4 studies provided AUROC values (median 0.79, range 0.69-0.93). Overall, HA was better at excluding severe fibrosis/cirrhosis rather than confirming it, and better in the detection of cirrhosis compared to milder degrees of fibrosis. In particular, in the study of Plevris *et al*[39] including 70 alcoholic patients, a threshold of HA > 100 μg/L had a 89% specificity and 87% sensitivity for diagnosing cirrhosis, with the specificity increasing to 96% for cut-offs of > 300 μg/L, thus reliably ruling out cirrhosis despite the small number on patients considered. Conversely, Tran *et al*[40] considered a lower threshold of HA (60 μg/L) in 146 heavy drinkers and showed a sensitivity of 100% and a specificity of 86% for the detection of cirrhosis. These data suggest that HA could be used as a screening test in patients who abuse alcohol in order to exclude advanced liver disease, provided that a standardized cut-off is adequately validated. Conversely, Lieber[41] evaluated the performance of TIMP1, P3NT and HA among other markers, in a cohort of 247 pre-cirrhotic alcoholic patients. The study reported poor accuracy of all these markers in the prediction of advanced fibrosis, with

corresponding AUROCs of 0.68, 0.67 and 0.69. Plasma YKL-40 was tested in 146 heavy drinkers and its levels increased in parallel with the severity of fibrosis and inflammation. Considering a threshold of 330 mcg/L, YKL-40 had a sensitivity of 50.8% and a specificity of 88.5 for the detection of advanced fibrosis[42]. Therefore, direct serum markers in isolation are inadequate for the diagnosis of pre-cirrhotic fibrosis stages.

A non-commercial panel of indirect and direct non-invasive markers was recently developed to stratify the risk of advanced liver disease and liver-related complications in a cohort of 1038 patients with ALD in primary care. The panel consisted of HA, P3NP and platelet count, and classified patients as high risk (red group), intermediate-risk (amber group) and low-risk (green group). After a mean follow-up of 46 mo, no patients in the green group decompensated or died, compared to 3.3% and 14% of deaths in the amber and red group respectively. The AUROC of this panel was 0.78 and 0.81 for diagnosing any degree or significant fibrosis respectively. Since the red and amber groups had a significant reduction in survival compared to the green one, the panel could be used as a screening test in primary care to guide further secondary care referrals[43].

FibroTest® consists of total bilirubin, haptoglobin, GGT, α2 macroglobulin and Apolipoprotein A1. Imber-Bismut firstly tested its accuracy in 339 HCV-infected patients and reported an AUROC of 0.837 for the detection of significant fibrosis. In a cohort of 221 patients with ALD, FibroTest® values were significantly different among distinct stages of liver fibrosis (except between F0 and F1). In contrast, HA values only differed between advanced stages of fibrosis and F0-F2. Moreover, for ≥ F2, the AUROCs of FibroTest® and HA were 0.84 and 0.79 respectively, while for the diagnosis of cirrhosis the corresponding AUROC were comparable at 0.95 and 0.93 respectively[44].

HepaScore® combines age, gender, bilirubin, GGT, HA, and gamma2-macroglobulin into a score with values ranging from 0 to 1. In a cohort of 512 HCV patients, HepaScore® had acceptable diagnostic accuracy both for significant fibrosis (AUROC = 0.81) and cirrhosis (AUROC = 0.88)[29,45].

The FibroMeter® derives from the combination of several serum markers, such as platelet count, prothrombin index, transaminases, GGT, α2 macro-globulin, HA, blood urea, ferritin, age and sex. It comprises six different tests, one for traditional histological staging and one for fibrosis quantification for each of ALD, NAFLD and viral hepatitis, showing the highest performance in chronic C hepatitis[46]. In a cohort of 478 patients, of which 95 had ALD, Fibrometer® showed a better AUROC (0.96) in diagnosing F2-F4 fibrosis stage in ALD, compared to Forns index, APRI and FibroTest®[47].

In a study by the group that developed FibroTest®, its diagnostic accuracy was compared to that of FibroMeter® and Hepascore® in 218 patients with ALD. Significant correlations between fibrosis stages and each serum panel were found with no significant differences in their AUROC for the diagnosis of advanced fibrosis and cirrhosis, with corresponding values of 0.83 and 0.92-0.94. Nevertheless, in the multivariate analysis, FibroTest® was the only factor independently associated with both advanced fibrosis and cirrhosis, whereas Fibrometer® was independently associated only with cirrhosis. Interestingly, the combination of these indexes did not improve the diagnostic performance of FibroTest®. Moreover, all three scores were predictors of 5 and 10 year overall survival and non liver-related deaths equally well correlated with histological semi-quantitative staging. Once again, at multivariate analysis only Fibrotest® remained an independent prognostic factor of liver-related mortality[36].

ELF™ score was developed by Rosenberg *et al*[48] in 1021 patients with chronic liver disease of mixed aetiologies, showing a high accuracy for the detection of significant fibrosis. Importantly, it performed well in either hepatitis C, NAFLD or ALD; although only 64 patients had ALD in the initial cohort, the sensitivity and specificity for diagnosing significant fibrosis were 93% and 100% respectively. These promising results need further validation in a larger cohort of patients with ALD.

All proprietary serum panels will require independent validation in ALD by groups not involved in their development.

Imaging techniques: Data on transient elastography (TE) are limited in patients with ALD compared to other aetiologies of liver disease. Mueller has shown that the presence of steatohepatitis an/or ongoing alcohol abuse can result in falsely elevated stiffness measurements and suggested that TE should not be performed in patients with AST > 100 IU/L who are actively drinking[49,50].

Similar to other aetiologies of liver disease, there are no validated cut-offs for the diagnosis of specific histological stages in patients with ALD. Nguyen-Khac[34] studied 103 patients with ALD and showed that stiffness cut-off values of 7.8 and 11 kPa were predictive of ≥ F2 and ≥ F3 respectively, with corresponding sensitivity 80% and 86.7% and specificity 90.5% and 80.5%, whereas a cut-off of > 19.5 kPa was suggestive of cirrhosis. Similar results were reported in a study of 711 patients with chronic liver disease, of whom 89 (12.5%) had ALD, where a cut-off value of 17.6 kPa had a sensitivity of 77% and a specificity of 97% for the detection of cirrhosis[51]. In another cohort of 147 alcoholic patients, the AUROC for patients with advanced fibrosis and with cirrhosis were 0.94 and 0.87 respectively. Cut-off values of 12.9 kPa and 22.6 kPa were optimal for the diagnosis of ≥ F3 and F4 respectively[52].

A Cochrane meta-analysis retrieved only 7 studies

on patients with ALD with a total of 834 patients[50]. Although stiffness cut-offs varied among studies and were not defined a priori, the most commonly used cut-offs for the detection of advanced fibrosis and cirrhosis were 9.5 kPa and 12.5 kPa respectively. At these values, TE had a sensitivity of 92% and 95%, and specificity of 70% and 71% for the diagnosis of F3 and F4 respectively. Only one study reported on F1, therefore the performance and cut-off values of TE in mild disease are yet to be established. The wide range of cut-off values for diagnosing specific fibrosis stages significantly affected the specificity of TE. Therefore, the diagnostic accuracy of TE is lower in ALD than in other causes of liver disease, particularly HCV[53]. As previously suggested[50], TE was better in ruling out rather than confirming advanced fibrosis and cirrhosis, with negative likelihood ratios of 0.11 and 0.07 respectively. Before TE can be used in clinical practice, cut-off values need to be sufficiently validated both in patients who continue to abuse alcohol and abstainers.

In order to improve the performance of TE, its combination with FIB4 has been tested in a cohort of 418 patients, showing an improvement in the accuracy in detecting advanced liver fibrosis compared to the performance of each test alone. Indeed, the new score had a sensitivity of 92% and a specificity of 78% compared to 87% and 76% of TE alone[54]. No further data on the combination of non-invasive tests are specifically available in ALD.

ARFI has also been studied in patients with ALD. In a cohort of 99 alcoholic patients, ARFI was significantly better than APRI, with AUROC of 0.875 and 0.893 for diagnosing ≥ S3 and S4 stages of the Scheuer scoring system. The optimum cut-off values for ARFI were 1.40 m/s for S3 and 1.65 m/s for S4. Interestingly, cut-off values decreased in the presence of normal transaminases, suggesting an influence of liver inflammation on ARFI values similar to TE[55].

A recent study by Cassinotto *et al*[26] compared the performance of Fibroscan®, ARFI and SSI in 349 patients with mixed aetiology of liver disease using histology as the gold standard. It demonstrated a superior accuracy of SSI for diagnosing significant fibrosis (≥ F2) compared to ARFI and for diagnosing severe fibrosis (≥ F3) compared to Fibroscan®. Although there were patients with ALD included, no separate analysis was performed according to the aetiology of liver disease. Nevertheless, the comparative accuracy of the technique is not expected to differ according to the underlying aetiology of liver disease.

Bensamoun *et al*[56] compared the diagnostic accuracy of MRE and Fibrometer® in 90 patients with ALD, using TE as the gold standard for fibrosis assessment. The analysis revealed that MRE performed well in the diagnosis of ≥ F1, ≥ F3 and F4, with corresponding AUROCs of 0.94, 0.98 and 0.99; conversely, Fibrometer® could only accurately detect cirrhosis (F4) with an AUROC of 0.95, with lower values for ≥ F1, ≥ F2 and ≥ F3 respectively (0.63, 0.69 and 0.83). As TE has a high number of false positive reading in patients with ALD, it is difficult to interpret the results of this study as liver biopsy was not performed.

CONCLUSION

Currently, although several non-invasive fibrosis markers have been suggested as alternatives to liver biopsy in patients with ALD, none has been sufficiently validated. In contrast to other aetiologies of liver disease, most notably HCV, data are scarce and derive from small cohorts of patients. Indeed, two separate cost-effectiveness analyses of non-invasive fibrosis tests in ALD concluded that such tests cannot be currently recommended for the investigation and management of such patients[57,58]. This was based on both the lack of specific treatment for ALD other than abstinence and the limited data on the diagnostic accuracy of non-invasive fibrosis tests. As described in other liver diseases, the diagnostic accuracy of non-invasive tests in ALD is acceptable for the diagnosis of significant fibrosis or cirrhosis but not for lesser fibrosis stages.

Simple panels that rely on AST, such as APRI and AST/ALT ratio, are of reduced utility in ALD due to the higher AST values in such patients that do not necessarily correlate with severe fibrosis. Proprietary panels such as Fibrotest, Fibrometer and ELF score need further validation. HA has been used either alone or in non-proprietary panels[43], however the cut-offs used for the diagnosis of advanced fibrosis and/or cirrhosis vary across studies and therefore cannot be applied in everyday clinical practice.

The most widely evaluated non-invasive test is Fibroscan, however its specificity is suboptimal and can only be used for ruling out the presence of ≥ F3 or cirrhosis. Therefore, its use as a community-screening tool in patients who abuse alcohol should be further explored. Indeed, the use of non-invasive tests could be tailored to first tier screening of patients at risk, in order to diagnose early patients with progressive liver disease and offer targeted interventions for the prevention of decompensation.

In conclusion, even though evidence supporting the reliability and utility of non-invasive assessment of liver fibrosis in ALD is accumulating, sufficient validation of these tests is still lacking, whereas their accuracy for the detection of earlier stages of fibrosis needs to be improved.

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TOPIC HIGHLIGHT

2015 Advances in Nonalcoholic fatty liver disease

Endocrine causes of nonalcoholic fatty liver disease

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease in the industrialized world. The prevalence of NAFLD is increasing, becoming a substantial public health burden. NAFLD includes a broad spectrum of disorders, from simple conditions such as steatosis to severe manifestations such as fibrosis and cirrhosis. The relationship of NAFLD with metabolic alterations such as type 2 diabetes is well described and related to insulin resistance, with NAFLD being recognized as the hepatic manifestation of metabolic syndrome. However, NAFLD may also coincide with endocrine diseases such as polycystic ovary syndrome, hypothyroidism, growth hormone deficiency or hypercortisolism. It is therefore essential to remember, when discovering altered liver enzymes or hepatic steatosis on radiological exams, that endocrine diseases can cause NAFLD. Indeed, the overall prognosis of NAFLD may be modified by treatment of the underlying endocrine pathology. In this review, we will discuss endocrine diseases that can cause NALFD. Underlying pathophysiological mechanisms will be presented and specific treatments will be reviewed.

Key words: Endocrine diseases; Nonalcoholic fatty liver disease; Insulin resistance; Obesity; Type 2 diabetes

Core tip: The review discusses the links between nonalcoholic fatty liver disease and endocrine diseases, from common ones such as type 2 diabetes and polycystic ovary syndrome to rare disorders such as growth hormone deficiency. The pathophysiological mechanisms underlying these associations are described.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most

common liver disease in the Western world. The term "nonalcoholic steatohepatitis" (NASH) was introduced by Ludwig in 1980 following observations of patients, mainly obese women, with histological evidence of alcoholic hepatitis on liver biopsy without a history of alcohol abuse[1]. The term "NAFLD" was introduced in 1986 to define a spectrum ranging from hepatic steatosis to fibrosis and cirrhosis.

Given the strong association of NAFLD with metabolic syndrome and the worldwide epidemic of obesity, the prevalence of NAFLD is constantly increasing. In the United States, one-third of the overall population has NAFLD and 2%-5% have NASH[2]. Within the NAFLD spectrum, only patients with histologically proven NASH develop progressive liver disease. Progression is more likely in the setting of diabetes, insulin resistance (IR) and other pre-existing conditions[3].

As we will discuss in this review, the physio-pathological mechanism common in both NAFLD and many different endocrine diseases is IR. For this reason, it is important for endocrinologists and gastroenterologists to remember that NAFLD and endocrine diseases may coexist (Figure 1).

EPIDEMIOLOGY

In the United States, the prevalence of NAFLD varies between 10% and 35%[2], depending on the population studied and the modality used for diagnosis. Ultimately, liver biopsy is required to make a definitive diagnosis of NASH, and estimates from biopsy series indicate that the prevalence of NASH in the United States is between 2% and 5%. NAFLD linked to metabolic syndrome is the most common cause of NASH, but NAFLD may be found in association with other diseases (*e.g.*, Wilson disease, disorders of lipid metabolism, *etc*).

NAFLD is not unique to Western countries. NAFLD is also prevalent in developing countries[4], and data from the rest of the world suggest that the prevalence of NAFLD varies between 6% and 35%, with a median of 20%[3,5]. Most studies indicate that NAFLD is usually associated with metabolic syndrome, but studies in Asian countries also report NAFLD in non-obese individuals[6-9]. However, these findings may be explained by the fact that, for a given body mass index (BMI), body fat content is usually higher in Asians than in westerners[10].

Several cohorts have shown that NAFLD prevalence depends on ethnicity. Notably, Hispanics have the highest prevalence of NAFLD, hepatic steatosis, and elevated aminotransferases levels, followed by non-Hispanic whites, whereas African Americans have the lowest prevalence[5]. However, in the absence of liver biopsies, the true prevalence of NAFLD cannot be accurately estimated, and it is therefore difficult to draw clear conclusions from these analyses. Moreover, the recent MESA (Multi-Ethnic Study of Atherosclerosis) found no association between ethnicity and NAFLD[11].

NAFLD may be affected by genetic or environmental factors. Notably, 38% of Asian Indian men with the apolipoprotein C3 gene variant alleles C-482T and T-455C have NAFLD (compared to 0% amongst wild-type homozygotes). An association between these variant alleles, NAFLD and IR was therefore reported[12]. Recently, a nonsynonymous genetic variant (rs58542926) within the transmembrane 6 superfamily member 2 (*TM6SF2*) gene of unknown function was associated with the severity of NAFLD[13].

In summary, estimates of the prevalence of NAFLD should be considered with caution, as they may vary depending on the criteria used for diagnosis.

DIAGNOSIS

NAFLD encompasses a spectrum of diseases of different etiologies ranging from fat accumulation (steatosis) to inflammation and fibrosis (NASH) and finally cirrhosis. Formally, a diagnosis of NAFLD requires a liver biopsy with a lipid content of at least 5% of hepatocytes. In 20%-25% of cases, steatosis will evolve to NASH and, in turn, 20% of these patients will develop cirrhosis[14]. We will briefly discuss the different diagnostic methods.

Liver biopsy is the current gold standard for NASH diagnosis and staging[5], but the method is invasive and cannot be used in population-based studies. Only biopsy can assess inflammation and fibrosis. However, sampling variability may alter the accuracy of the diagnosis[15]. Several noninvasive diagnostic methods for NAFLD and NASH have been introduced recently. Notably, imaging techniques including proton magnetic resonance spectroscopy (^{1}H-MRS), ultrasonography, computed tomography (CT), and magnetic resonance imaging (MRI) can be used[16]. ^{1}H-MRS is considered the most accurate noninvasive method for measuring liver fat content. Ultrasonography is the most widely used method but is relatively insensitive, as it can detect steatosis only when liver fat content exceeds 33%[17].

Other studies have used elevations in the liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) as indicators of NAFLD[18-22]. However, these measurements are neither sensitive nor specific[23]. Indeed, up to 70% of subjects with NAFLD have normal levels of ALT and AST[17].

Different scoring methods have been developed for NAFLD screening, such as the Fatty Liver Index[24] and the Lipid Accumulation Product[25]. These indices are easy to use, applicable in community healthcare settings, and could contribute to better assess NAFLD prevalence. A study published by the LIDO study group tried to validate five NAFLD scoring methods (fatty liver index, NAFLD liver fat score, hepatic steatosis index, visceral adiposity index and triglyceride × glucose index) in patients with biopsy-confirmed NAFLD. All of these methods diagnosed hepatic steatosis but failed

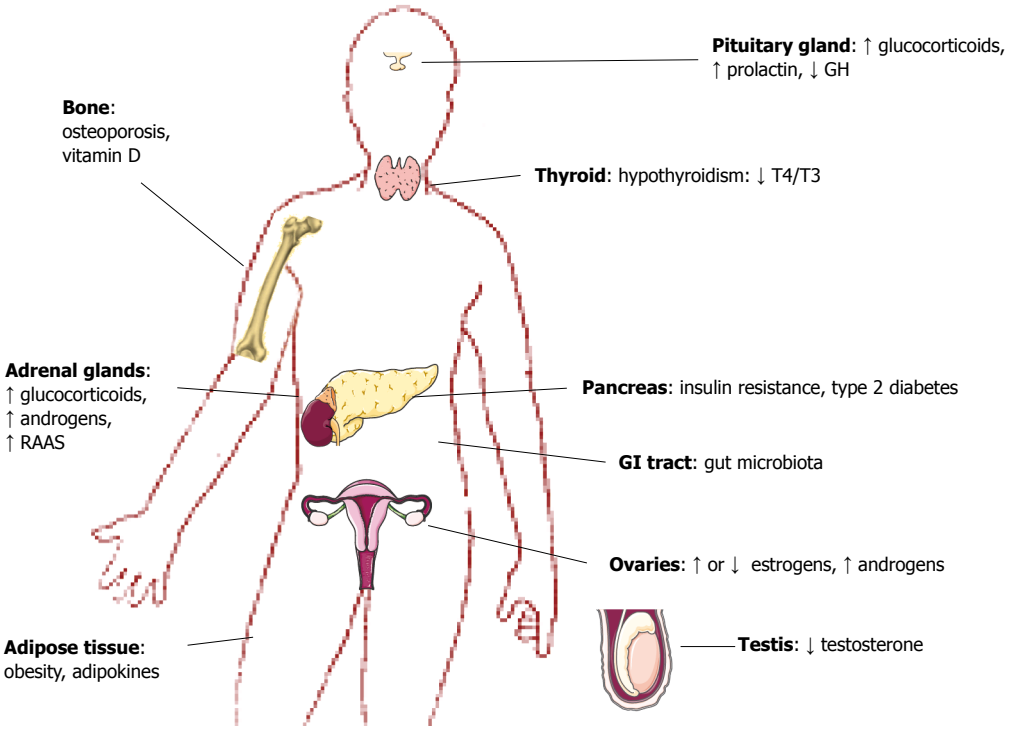

Figure 1 Endocrine diseases associated with nonalcoholic fatty liver disease. GH: Growth hormone; RAAS: Renin-angiotensin-aldosterone system; GI: Gastrointestinal.

to quantify the severity[26].

More specific scoring methods using other biomarkers, such as α-2-macroglobulin, haptoglobin, apolipoprotein a1, and γ-glutamyl-transferase, have to be developed in order to better select patients for liver biopsy[27].

Clinicians should consider NAFLD in a patient with abnormal liver tests and at least one metabolic risk factor. However, clinical features are nonspecific and patients are usually asymptomatic until they progress to liver cirrhosis.

PATHOGENESIS

Historically, liver injury is thought to be the result of the "two-hit hypothesis" involving IR and altered adipokine production, resulting in oxidative stress and apoptosis[28] (Figure 2).

The "two-hit hypothesis" was first described by Day *et al*[29] in 1998. The first hit represents accumulation of triglycerides (TG) and free fatty acids (FFA) from visceral adipose tissue in hepatocytes secondary to IR. FFA are transported to organs including the liver and undergo either β-oxidation in the mitochondria or are stored as TG. TG stored in the liver come principally from lipolysis of white adipose tissue, but also from dietary lipids and *de novo* lipogenesis[30]. If an imbalance is present, excessive FFA flux and accumulation induce hepatic IR.

Once hepatic steatosis is established, progression to steatohepatitis involves a "second hit", consisting of inflammation, mitochondrial dysfunction, enhanced oxidative stress caused by reactive oxygen species, lipid oxidation and production of adipokines resulting in hepatocyte damage and fibrosis[29]. Fatty liver is susceptible to oxidative injury and lipid peroxidation[31].

In 2010 Tilg and Moschen[32] introduced the "multi-parallel hit" hypothesis to explain NAFLD pathogenesis. This hypothesis stresses the importance of gut-derived and adipose tissue-derived factors that promote liver inflammation and fibrosis. This hypothesis, based on reports that endoplasmic reticulum stress[33] and cytokine-mediated stress can induce steatosis as well as necroinflammation, suggests that multiple "hits" act together in parallel in the development of NASH[32]. The role of the gut microbiota in this process will be discussed below.

A more detailed discussion of NAFLD pathogenesis and its link with IR can be found elsewhere[34].

Gut microbiota

The gastroenterological tract contains more than 10^{14} microorganisms, including more than a thousand bacterial species. The role of gut microbiota in the pathogenesis of obesity is now being recognized. By regulating liver fat deposition and energy homeostasis, gut microbiota may also play a role in NAFLD pathogenesis.

The liver is supplied primarily by the portal system and is therefore exposed to metabolites originating from intestinal bacteria (such as ethanol and other volatile organic compounds) or the bacteria

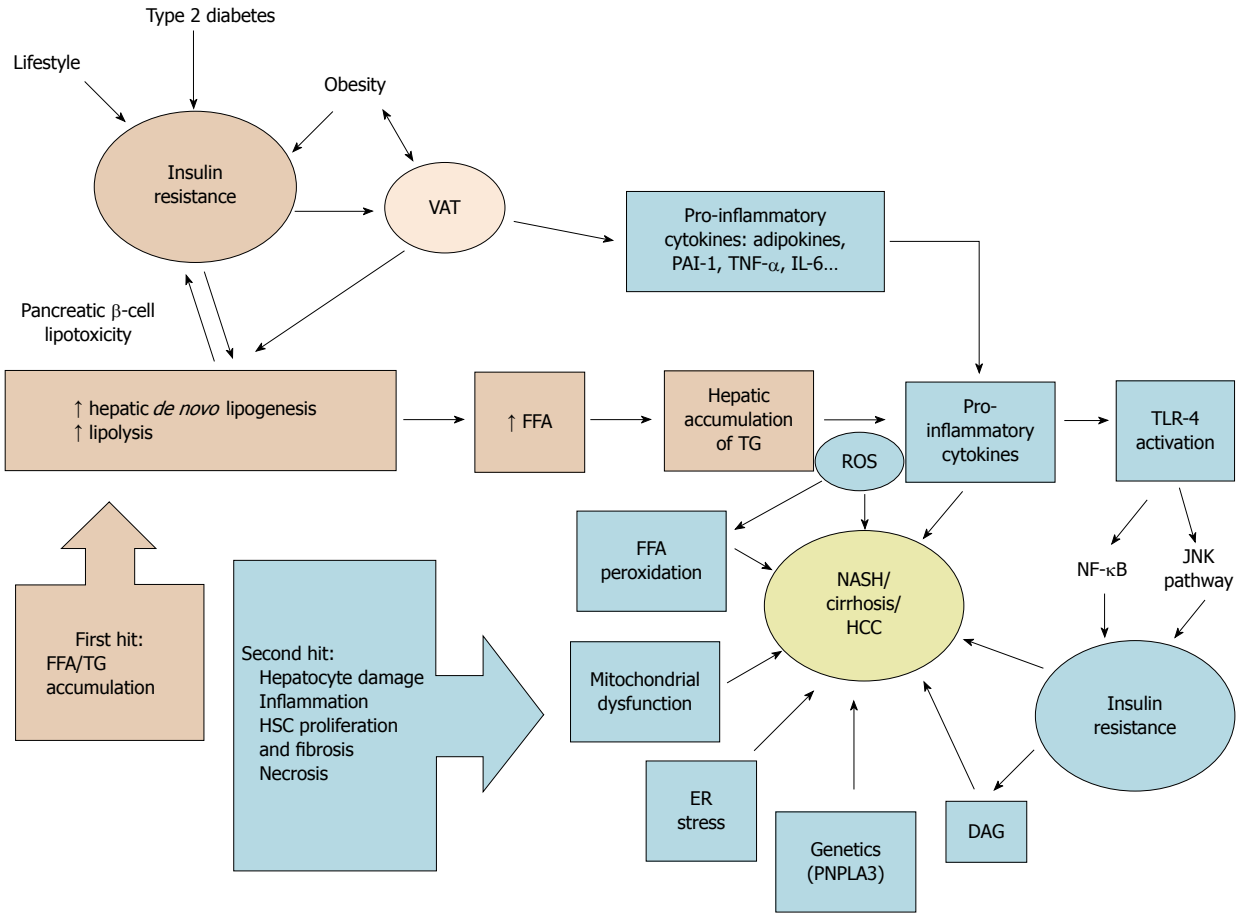

Figure 2 Schematic summary of nonalcoholic fatty liver disease pathophysiology according to the "two-hit hypothesis". VAT: Visceral adipose tissue; FFA: Free fatty acid; TG: Triglycerides; PAI-1: Plasminogen activator inhibitor-1; TNF-α: Tumor necrosis factor α; IL-6: Interleukin 6; ROS: Reactive oxygen species; TLR-4: Toll-like receptor 4; DAG: Diacylglycerols; ER: Endoplasmic reticulum; NASH: Non-alcoholic steatohepatitis; HCC: Hepatocellular carcinoma; PNPLA3: Patatin-like phospholipase domain-containing protein 3; NF-κB: Nuclear factor-kappa B; JNK: c-Jun N-terminal kinases; HSC: Hepatic stellate cells.

themselves[35]. The liver acts as a barrier between the gut and the systemic circulation by removing toxins. When Kupffer cells, the specialized macrophages in the hepatic sinusoids, are impaired, or when the gut-mucosal barrier is damaged by inflammation or portal hypertension, a metabolic endotoxinemia results. The high endotoxin level activates Kupffer cells and hepatic stellate cells (HSC). Bacteria can also produce lipopolysaccharides (LPS), which bind to Toll-like receptor 4 (TLR-4) and induce the production of pro-inflammatory cytokines[36], subsequently leading to inflammation. These events then contribute to the pathogenesis of obesity and NAFLD[37,38].

Patients with biopsy-proven NAFLD have increased gut permeability and small intestinal bacterial overgrowth, which play an important role in the alteration of hepatic fat metabolism[39]. In obese children with biopsy-proven NAFLD, expression of zonulin, a modulator of intracellular tight junctions, is increased in parallel with the severity of hepatic steatosis. However, there was no significant correlation of plasma zonulin concentrations with lobular inflammation, fibrosis or NASH[40]. These data have not been verified in adults.

Obese people have a different microbiota composition than lean people, with an increase in *Firmicutes* and a 50% decrease in *Bacteroidetes*[41,42]. This results in a change in short-chain fatty acids and an increase in intestinal energy absorption[43]. Patients with NAFLD also have different microbiota, with less *Bacteroidetes* and *Lactobacilli* and more *Prevotella* and *Porphyromonas* compared to healthy controls[44]. However, these findings are controversial with inconsistent data.

Together, bacterial overgrowth and increased intestinal permeability contribute to NAFLD pathogenesis[39]. Mouzaki *et al*[45], in a prospective cross-sectional study, assessed whether differences in gut microbiota could be associated with the development of NAFLD. The authors found that, independently of diet and BMI, NASH patients contained a lower ratio of *Bacteriodetes* to *Prevotella* than did healthy controls. In contrast, Raman *et al*[46], in an observational case-control study of obese patients with NAFLD *vs* healthy controls, found that *Bacteriodetes* representation was similar between the two groups. Interestingly, gut microbiota might contribute to the development of NAFLD through ethanol production[47]. Further studies are needed to clarify whether gut

microbiota contributes to NAFLD pathogenesis or if representational differences are a result of the disease. Nevertheless, gut microbiota affects the susceptibility to NASH *via* metabolic endotoxinemia mediated by bacterial ethanol production, alterations in choline and bile acid metabolism, hepatocyte lipogenesis and increased intestinal permeability[43].

Probiotics modulate intestinal flora and have been proposed as a beneficial complement to NAFLD treatment[48]. Probiotics modulate gut microbiota, reduce inflammation, increase epithelial barrier function, and increase antibacterial substance production[35]. A meta-analysis of four randomized clinical trials showed that probiotic therapy decreases plasma levels of aminotransferases, total cholesterol and HDL cholesterol, and improves the Homeostasis Model Assessment of insulin resistance (HOMA-IR) index[49]. However, these studies were conducted with small group sizes without dietary control. The results should therefore be considered with caution, and the use of probiotics for NAFLD is not recommended at this time[50].

PROGNOSIS

Studies based on histological data suggest that only patients with NASH are at risk of disease progression[27]. Patients with NAFLD are, however, prone to develop type 2 diabetes. In a Swedish cohort study, most patients with NAFLD (78%) were diagnosed with diabetes or impaired glucose tolerance at follow-up. Progression to liver fibrosis occurred in 41% of the patients and was associated with marked IR and pronounced weight gain[51]. A major prognostic issue in NAFLD is hepatocellular carcinoma. Finally, NAFLD is associated with cardiovascular diseases and has emerged as a new cardiovascular risk factor (see below).

Liver transplantation is the treatment for end-stage liver disease. However, *de novo* NAFLD after transplantation has been reported to be common: in a retrospective study, 75% of the patients developed fatty infiltration of the graft and 38% developed NASH[52].

METABOLIC CONSEQUENCES: CARDIOVASCULAR DISEASE

NAFLD increases the incidence of cardiovascular disease (CVD) and is a predictor of CVD of other risks factors[53]. Accordingly to the review of Edens *et al*[54], NAFLD is linked to the CVD risk profile. After adjusting for cardiovascular risk factors, NAFLD is independently associated with markers of subclinical atherosclerosis such as impaired flow-mediated vasodilation, increased carotid artery intima-media thickness and arterial stiffness[55]. NAFLD patients are more likely than healthy individuals to have advanced high-risk coronary atherosclerosis, correlated with the severity of hepatic fibrosis[56]. Moreover, the presence of hepatic fibrosis is predictive of cardiovascular events[57]. The coronary artery calcium score is often used as a surrogate marker of coronary atherosclerosis and is considered an independent predictor of CVD[58]. Fatty liver and HOMA-IR are each associated with a high coronary artery calcium score (37.9% and 26.0%, respectively)[59]. In the MESA study, NAFLD was associated with high coronary artery calcium scores and inflammation independently of obesity and metabolic syndrome[11]. Recently, in the "Hepatic steatosis and cardiovascular disease outcomes" sub-analysis of the Framingham Heart study including 3014 participants, there was a significant association of hepatic steatosis with coronary artery calcium score. However, there was a non-significant association between hepatic steatosis and clinical CVD (non-fatal myocardial infarction, stroke, transient ischemic attack, heart failure or peripheral arterial disease)[60]. Interestingly, the increase in cardiovascular events in patients with NAFLD is almost always associated with diabetes[61-63]. NAFLD is frequently associated with dyslipidemia (high triglycerides, low HDL, high VLDL) and increased levels of pro-inflammatory cytokines which are atherogenic[64] and promote the development of CVD[65]. Finally, hepatokines such as fibroblast growth factor 21 (FGF21), fetuin-A and selenoprotein P may also play a role in the development of CVD[66].

ENDOCRINE DISEASES ASSOCIATED WITH NAFLD

Type 2 diabetes

NAFLD is more prevalent in patients with pre-existing metabolic conditions than in the general population. Specifically, type 2 diabetes and NAFLD have a particularly close relationship. A cross-sectional study of patients under 65 with type 2 diabetes found a 69% prevalence of ultrasonographic NAFLD[67] , and the prevalence varies from 30% to 70% in other studies[68,69]. In an Indian cohort, 127 of 204 diabetic patients displayed fatty liver on ultrasound. Among these, 87% were diagnosed with NAFLD after a liver biopsy[70]. Therefore, the prevalence of NAFLD is higher in patients with type 2 diabetes than in the general population, IR being the central mechanism of both diseases.

In addition to having a higher prevalence, liver disease may be more progressive in patients with type 2 diabetes. Diabetic patients with elevated BMI are at higher risk for fibrosis progression[71]. Even without diabetes, IR is a hallmark for cirrhosis[72]. A significant and independent association of degree of IR and stage of fibrosis suggests that severe IR may contribute to fibrosis development in NAFLD[14,73]. Consistent with IR, patients with NAFLD have reduced insulin sensitivity in muscle, liver and adipose tissue[74]. Finally, glucose

intolerance or type 2 diabetes is found in 20%-70% of patients with NASH[75,76].

Obesity

Here the prevalence of NAFLD ranges from 57% in overweight individuals attending outpatient clinics to 98% in nondiabetic obese patients[77-79]. The median prevalence of NASH in the obese population is 33%, ranging from 10% to 56%[79-81]. Bariatric surgery is becoming a frequent treatment option and intra-operative liver biopsies are now frequently performed. For example, in a study by Boza *et al*[80], the prevalence of NAFLD and cirrhosis in a cohort of obese patients undergoing gastric bypass surgery was 63% and 2%, respectively.

In obesity, visceral fat contributes to IR by liberating FFA that accumulates in the liver[82]. Hepatic fat content is correlated with IR as well. In some studies, hepatic fat content cancelled the correlation of visceral fat with IR[83], but in other studies there was an independent contribution of both visceral fat and IR to hepatic fat content[84]. Interestingly, perivascular and epicardial lipid deposits are correlated with atherosclerosis and metabolic syndrome[85]. Moreover, epicardial lipids are correlated with visceral fat, coronary artery disease, presence of NAFLD, and even the severity of liver fibrosis[86,87].

Due to age-related changes in body fat distribution, especially an increase in visceral fat, the prevalence of NAFLD increases with age[88]. Visceral adipose tissue produces FFA and diverse adipokines involved in NAFLD pathogenesis such as increased levels of tumor necrosis factor-α (TNF-α), resistin, and interleukin-6 (IL-6) and decreased levels of adiponectin[89]. Hypertrophied adipocytes promote, *via* adipokine secretion, accumulation of macrophages in the visceral fat. These macrophages produce pro-inflammatory cytokines, resulting in chronic inflammation that further exacerbates IR[90].

Altogether, these data reveal a central role of obesity in the development of IR and NAFLD.

Adipokines: Adipokines are cytokines secreted by adipose tissue that are involved in adipose homeostasis and lipid metabolism. Many adipokines are being studied as potential targets for new drugs. Adiponectin, ghrelin and leptin are adipokines that decrease IR, while TNF-α and IL-6 are cytokines that enhance IR and, subsequently, NAFLD[91]. However, IL-6 can play either a pro- or an anti-inflammatory role[92,93].

Leptin: Leptin is a protein encoded by the *ob* gene and produced mainly by adipocytes, but also by the skeletal muscle, stomach, ovaries and liver[94]. This peptide plays an anorexigenic role in the regulation of body weight, acting on the hypothalamus to decrease appetite and increasing energy expenditure *via* sympathetic stimulation of several tissues. The anti-lipogenic effect of leptin is mediated by lowering the expression of sterol regulatory element binding protein 1, which regulates genes involved in *de novo* lipogenesis[95]. Leptin down-regulates pre-proinsulin transcription and insulin secretion, explaining why leptin levels are high in insulin-resistant patients[91]. Leptin production is stimulated by pro-inflammatory cytokines (*e.g.*, IL-1, TNF-α)[96]. Expression of leptin in visceral adipose tissue is associated with NAFLD features[97]. Leptin participates in NASH not only *via* IR but also perhaps in the regulation of HSC, contributing to the development of hepatic fibrosis[98]. *In vitro*, leptin has a fibrogenic effect on HSC[99] by an unknown mechanism. Mice deficient in leptin signaling are obese and have increased lipid accumulation in liver[100], and leptin infusion in wild-type mice attenuates hepatic steatosis and hyperinsulinemia[101]. In clinical studies, leptin levels are elevated in patients with NASH and correlate with fibrosis severity[102]. However, in some studies this association disappears when leptin levels are adjusted for variables such as age, gender, BMI and hyperinsulinemia, all of which influence leptin levels[14,103].

Adiponectin: Adiponectin, an anti-inflammatory cytokine, is produced predominantly by adipocytes at a level inversely correlated with visceral fat content. Low adiponectin levels are associated with IR and type 2 diabetes, dyslipidemia, hypertension, and NAFLD[104-107]. In animal studies and *in vitro*, adiponectin exhibits an anti-inflammatory effect by impairing NF-κB activity and inhibiting TNF-α-induced expression of endothelial adhesion molecules. Moreover, adiponectin decreases LPS-induced TNF-α production[108-110]. Anti-oxidative, anti-steatotic and anti-fibrotic effects have also been demonstrated[111]. Indeed, disruption of adiponectin receptors increases tissue triglyceride content, inflammation, oxidative stress and IR[112]. Adiponectin can prevent lipid accumulation in patients with NASH by increasing β-oxidation and by decreasing synthesis of FFA in hepatocytes[113].

In human studies, high plasma levels of adiponectin are correlated with a decreased risk of developing type 2 diabetes[114], and lower adiponectin levels have been shown to be an independent risk factor for NAFLD[115]. Adiponectin levels are correlated with NAFLD progression and are therefore a prognostic factor[116,117].

Endocrine disruptors

Endocrine disruptors (EDCs) are becoming an important health- and environment-related concern. Recent studies indicated that exposure to bisphenol A *in utero* increases the likelihood of adulthood hepatic steatosis by altering hepatic β-oxidation capacity, possibly through epigenetic mechanisms[118,119].

EDCs (dioxins, phtalates, bisphenol A, persistent

organic pollutants) may induce IR, either directly by increasing oxidative stress or indirectly by altering gene transcription, *e.g.*, down-regulating adiponectin[120]. For example, high bisphenol A levels are associated with increased IR and hepatic steatosis[121]. However, the time gap between fetal exposure and adult disease manifestation makes the causal relationship difficult to prove. A systematic review of observational studies demonstrated an association between EDCs and NAFLD but failed to demonstrate causality. Interventional mechanistic studies (reducing or eliminating EDC exposure) are difficult to conduct but are essential for determining the role of EDCs in NAFLD pathogenesis[122].

Sexual hormones

Polycystic ovary syndrome: Polycystic ovary syndrome (PCOS) is an endocrine syndrome frequently encountered in young women of childbearing age (prevalence 8%-15%)[123,124], hallmarked by clinical and/or biological hyperandrogenism, oligo/amenorrhea and polycystic ovarian morphology following ultrasound[124].

Genes influencing obesity and IR, β-cell dysfunction, steroid production and metabolism, androgen receptor and X-inactivation, and ovarian folliculogenesis have been studied as candidates for PCOS pathogenesis[125]. Genowe-wide association studies conducted in women with PCOS have found a relationship between the syndrome and several genes involved in type 2 diabetes, such as *THADA*, *INSR* and *HMGA2*[126]. In European populations, the *DENND1A* variant is associated with hyperandrogenism and oligomenorrhea[126]. IR occurs in about half of women with PCOS[127]. A recent meta-analysis from Ramezani-Binabaj *et al*[128] showed that there is a higher risk of NAFLD among women with PCOS (overall OR = 3.93). The prevalence of NAFLD in women with PCOS is between 15% and 55%[129-131], depending on the diagnostic method used. Conversely, the prevalence of PCOS in women with NAFLD is high as well, 71% in one cohort[132].

However, it is not clear whether PCOS is an independent risk factor for NAFLD. Gambarin-Gelwan *et al*[130] studied lean and obese women with PCOS and found a NAFLD prevalence of 39% in the lean group. Steatosis was associated with a higher BMI and HOMA-IR and a higher prevalence of glucose intolerance and type 2 diabetes. Moreover, women with PCOS had more IR than control women with the same BMI (Figure 3).

IR is a major player in PCOS, promoting hyperandrogenism *via* an increased release of androstenedione and testosterone[133]. Insulin acts as a co-gonadotropin to increase luteinizing hormone-stimulated production of androgens; therefore, production of androgens is enhanced in PCOS. A concomitant decrease in sex hormone binding globulin (SHBG) by impaired liver production amplifies this phenomenon, further increasing the levels of free and active androgens. The decrease in SHBG is mediated by IR and hyperandrogenism, further increasing IR. Different hypotheses have been raised to explain why IR is present in PCOS. It is suggested that post-receptor defects in insulin receptor signal transduction are involved, because no structural abnormality in the insulin receptor has been identified in these patients[134]. Impaired glucose transport has also been suggested[135]. Defective serine phosphorylation can lead to both IR and hyperandrogenism, given that serine phosphorylation modulates the activity of key regulating enzymes of androgen biosynthesis, including the 17, 20 lyase activities of P450c17[136,137].

Whether PCOS contributes independently to NAFLD is unclear. PCOS diagnosis is significantly associated with NAFLD, after adjustments are made for age, obesity, waist circumference[138] and dyslipidemia[139]. As hyperandrogenism is a hallmark of PCOS, androgens likely play a role in the development of NAFLD. It has been hypothesized that androgens promote a pro-apoptotic environment[140] which is present in woman with PCOS[141,142]. The caspase 3-cleaved fragment of cytokeratin 18 is released from cells undergoing apoptosis and is now established as a serum marker for NASH. The levels of this fragment correlate with hepatocyte apoptosis and is elevated in women with PCOS[142]. It is not clear whether hyperandrogenism and IR act concomitantly or independently to induce NAFLD, but a synergistic action may be present[143]. Interestingly, oophorectomy in a patient with hyperthecosis reverses hirsutism but not IR[144]. However, the link between hyperandrogenism and NAFLD may be found in the down-regulation of the LDL-receptor, prolonging the half-lives of VLDL and LDL, inducing accumulation of fat in the liver and ultimately triggering NAFLD[141]. In addition, women with hyperandrogenism have higher transaminases levels (predominantly ALT) compared to control subjects, even if the women with hyperandrogenism are lean[138]. Moreover, central obesity and visceral fat are often increased in PCOS women and can be involved in the development of both IR and NAFLD. Indeed, women with PCOS have larger adipocytes, with a diameter increased by 25% ("hypertrophic obesity"), compared to obese women without PCOS ("hyperplastic obesity")[145]. Hypertrophic obesity is associated with IR and can be mediated by androgens *in vivo*[146]. The role of leptin in PCOS is unclear. Compared with BMI-matched controls, lean PCOS patients have lower soluble leptin receptor levels, and PCOS *per se* might cause leptin resistance with higher free leptin indices[147]. Adiponectin levels are lower in women with PCOS (after controlling for BMI-related effects), in relationship with IR, but not in women with hyperandrogenism[148].

The association between PCOS and NAFLD is crucial to recognize considering the former's young

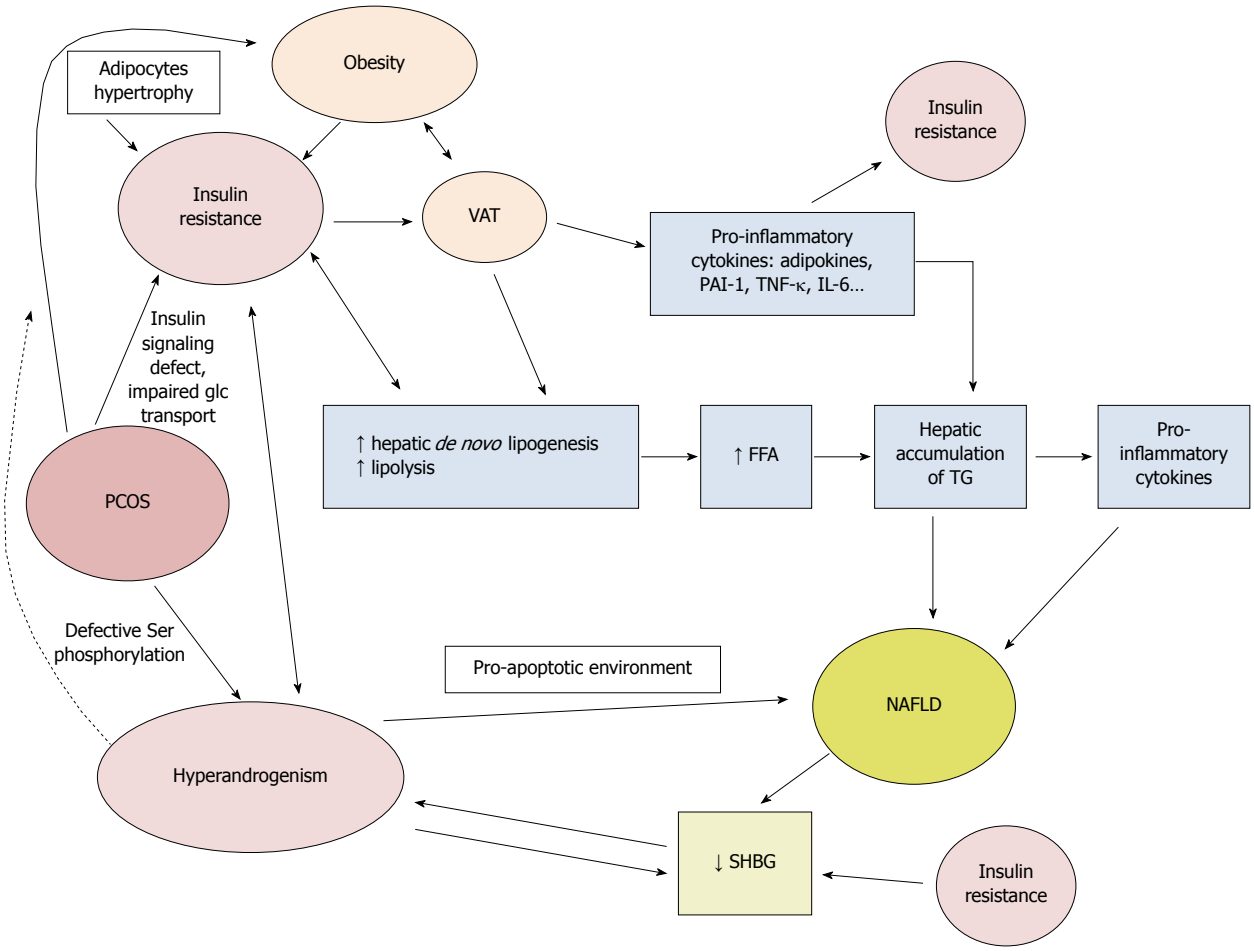

Figure 3 Pathophysiological mechanisms linking polycystic ovary syndrome and nonalcoholic fatty liver disease. Ser: Serine; VAT: Visceral adipose tissue; glc: Glucose; SHBG: Sex hormone binding globulin; FFA: Free fatty acids; TG: Triglycerides; PAI-1: Plasminogen activator inhibitor 1; TNF-α: Tumor necrosis factor α; IL-6: Interleukin 6.

presentation age. It is important to screen young women with PCOS and an associated metabolic syndrome or IR for NAFLD, although the best screening method has not been defined[149]. Routine screening is not recommended by the Endocrine Society[150], but screening high-risk patients seems reasonable. The use of the Fatty Liver Index may be helpful and can identify PCOS patients at high risk for hepatic disturbances[149].

Estrogen deficiency/menopause: Several studies indicate that estrogens play a protective role in NAFLD. NAFLD is more prevalent in post-menopausal women than pre-menopausal women and worsens after menopause[151]. Moreover, estradiol levels in women with PCOS are lower than in women without PCOS[152].

The effects of estrogens are mediated not only through activation of estrogen receptors (ER) α and β but also by non-nuclear activities[153]. Estrogens regulate growth hormone (GH) production and energy homeostasis[154]. In murine models, estrogens have been shown to suppress hepatic fibrosis by attenuating HSC activation[155]. In ERα knockout mice (αERKO) and aromatase knockout mice (ARKO), estrogens are either not synthesized or cannot act properly. These mice contain increased amounts of visceral adipose tissue, as well as an accumulation of lipid droplets in the liver of ARKO mice, highlighting the importance of estrogens in lipid homeostasis[156]. αERKO mice manifest adipocyte hyperplasia and hypertrophy, IR and glucose intolerance in both sexes[157], and steatosis in males[158]. Interestingly, patients with an aromatase gene inactivating mutation (aromatase deficiency) exhibit estrogen deficiency, development of metabolic syndrome with IR, steatohepatitis and precocious atherogenesis. When these patients undergo estrogen treatment, their IR and liver steatohepatitis improve[159]. Moreover, estrogen replacement therapy in mice has been shown to prevent diet-induced ectopic lipid (notably diacylglycerols) deposition as well as hepatic and muscle IR[160].

Male hypogonadism: Male hypogonadism includes biochemical and clinical features such as low testosterone and/or low sperm count, erectile dysfunction, diminished libido, decrease in lean body mass and increase in visceral fat, as defined by the International Society of Andrology, the International Society for the Study of the Aging Male and the European Association of Urology.

Testosterone plays a key role in insulin sensitivity, body composition and lipid metabolism[161]. A bidirectional relationship exists between low levels of testosterone and IR[161-163]. The HERITAGE study indicated that people with lower testosterone levels have a preferential accumulation of abdominal fat and a higher visceral adipose tissue accumulation[164]. Low levels of testosterone and SHBG in men are independent predictors of the occurrence of metabolic syndrome[165]. Men with metabolic syndrome have a higher prevalence of low testosterone compared to healthy controls[166,167]. Furthermore, according to the hypogonadal-obesity-adipokine hypothesis, increased amounts of adipose tissue converts testosterone to estradiol *via* aromatase activity. Estradiol inhibits kisspeptin liberation and testosterone production. Moreover, adipose tissue produces leptin and pro-inflammatory cytokines that both have an effect on the gonadal axis, impairing testosterone production. Leptin has an additional effect on Leydig cells, resulting in decreased androgen production[168].

In a retrospective cohort study, hepatic steatosis, defined by sonographic criteria, was correlated with low testosterone levels (< 14.2 nmol/L) after adjusting for diverse confounders (including age, BMI, smoking, diabetes, and visceral adipose tissue)[169]. A recent cross-sectional study using data from MESA study showed that men with the highest tertile of SHBG were less likely to have a fatty liver, defined by computed tomography, than those in the lower tertile[170].

Interventional studies using testosterone replacement therapy in hypogonadal men have shown that testosterone not only improved insulin sensitivity but also decreased waist circumference[171,172] together with BMI[173]. In obese men with sleep apnea, testosterone replacement therapy led to increased insulin sensitivity and reduced liver fat content[174]. In castrated rats on a high-fat diet, testosterone replacement therapy led to a lower body fat percentage and only mild-moderate microvesicular steatosis compared to castrated rats not receiving testosterone, which displayed severe micro- and macrovesicular fat in hepatocytes[175]. However, the evolution of NAFLD during testosterone replacement therapy in men has not been studied in clinical trials.

Osteoporosis

Evidence for an important triumvirate (NAFLD, osteoporosis and metabolic syndrome) is rising[176]. A complex crosstalk of mediators coming from the liver (fetuin-A), adipose tissue (leptin, TNF-α, adiponectin) and bone (osteopontin, osteocalcin, osteoprotegerin) may contribute to the development of NAFLD and metabolic syndrome[177], and the protective effect of obesity on bone mass is progressively challenged[178]. For example, insulin can increase bone formation by binding to the insulin receptor on osteoblasts, and leptin and adiponectin can suppress bone formation or stimulate resorption. Conversely, the bone also affects glucose metabolism, by secreting cytokines, hormones and peptides like osteocalcin which increase pancreatic β-cell function[179]. Mice lacking the insulin receptor on osteoblasts develop obesity and IR that are improved after osteocalcin administration, suggesting the presence of a bone-pancreas loop[180].

In post-menopausal women with an ultrasonographic diagnosis of NAFLD, lumbar bone mass density was found to be lower (0.98 ± 0.01 g/cm² *vs* 1.01 ± 0.02 g/cm², $P = 0.046$) than in controls, after adjusting for age, BMI, ALT levels, smoking and alcohol consumption. This phenomenon was also demonstrated after adjusting for metabolic syndrome[106]. Among Asian men, NAFLD (diagnosed by ultrasound) was significantly associated with osteoporotic fractures, defined as fractures secondary to low trauma[181]; however, the association did not reach significance in women[105].

Treatment of NAFLD may also have an impact on bone. Thiazolidinediones, which are peroxisome proliferator-activated receptor γ (PPARγ) agonists, improve insulin sensitivity and reduce hepatic fibrosis progression[179], but also increase bone loss and fractures, especially vertebral fractures in males with type 2 diabetes[182].

Further studies are needed to better understand the interactions between osteoporosis and NAFLD.

Vitamin D deficiency

The pleiotropic effects of vitamin D, particularly on metabolism and the immune system, are being increasingly studied. NAFLD has been associated with low 25-OH vitamin D levels. Notably, a recent meta-analysis found that NAFLD patients are 26% more likely to be deficient in vitamin D compared with controls[183]. However, the two conditions are quite frequent and the association may be fortuitous. The use of a cross-sectional approach and the method to diagnose NAFLD are two limitations of this study. Other studies found that 25-OH vitamin D levels can predict the histological severity of NAFLD, with NASH patients having lower levels than individuals with simple steatosis, even children[184,185]. However, in the study of Dasarathy *et al*[184], the control group was smaller in number, had a lower BMI and was not age-matched. These differences may influence vitamin D levels, as obese patients have lower vitamin D levels.

It is unclear how vitamin D could prevent or slow the development of NAFLD. However, vitamin D has been shown to inhibit the proliferation of HSC, which express the vitamin D receptor[186], and therefore could reduce the fibrotic process. The work of Roth *et al*[187] demonstrates in a rat model that phototherapy can reduce fibrosis, apoptosis and inflammation, primarily by reducing hepatic expression of inflammatory genes such as TNF-α and transforming growth factor β. The authors conclude that vitamin D deficiency exacerbates inflammatory gene expression and is partially

reversible[188]. In another study, treatment of adipocytes with calcitriol (1,25-OH-vitamin D) caused the GLUT4 transporter to be upregulated and translocated to the cell surface, resulting in increased glucose uptake and utilization[189]. A double-blind, placebo-controlled Iranian study[190] showed that vitamin D supplementation decreases the inflammatory marker hs-CRP, but there was no effect on liver enzymes, HOMA-IR or steatosis grade (evaluated by ultrasound). However, this study enrolled only 53 patients and was conducted for a short period of time.

Altogether, only a limited number of prospective and randomized studies have analyzed the impact of vitamin D supplementation on NAFLD. Therefore, the effect of vitamin D supplementation on NAFLD has to be further studied, as concluded in a recent review by Eliades *et al*[191].

Pituitary gland

Growth hormone insufficiency: GH and insulin-like growth factor-1 (IGF-1) insufficiency have recently been associated with NAFLD, progression to NASH and even liver cirrhosis. NAFLD is more common in hypopituitary patients than control subjects and patients with growth hormone deficiency (GHD) are likely to have an increased risk of developing NAFLD. In a Korean cohort of men with hypopituitarism, the frequency of NAFLD (diagnosed by abdominal ultrasonography) was significantly higher in hypopituitary men than in control subjects (32.5% *vs* 70.6%, $P = 0.001$). CRP and FFA were significantly elevated in hypopituitary patients with NAFLD compared to hypopituitary patients without NAFLD. Moreover, the severity of NAFLD correlated negatively with GH after adjusting for BMI ($P = 0.020$). Severe GHD in hypopituitarism was associated with more advanced NAFLD[192]. In one series NAFLD developed after 6.4 ± 7.5 years (median 3 years) in GHD patients[193].

GHD leads to visceral adiposity, reduced lean body mass, an abnormal lipid profile and IR[194]. However, the exact pathophysiological mechanisms need to be clarified[195]. Recent data show a relationship between low IGF-1 and Sirtuin4 (Sirt4) levels. Obese patients with low levels of GH or IGF-1 have a higher waist circumference and/or metabolic syndrome. Like GH, which regulates mitochondrial oxidative capacity, Sirt4 is a mitochondrial NAD-dependent ADP-ribosyltransferase that inhibits mitochondrial glutamate dehydrogenase 1 activity, thereby down-regulating insulin secretion in response to amino acids. Sirt4 functions within the mitochondria as a negative regulator of oxidative capacity. Levels of Sirt4 are low in obese patients, in order to preserve fat oxidative capacity and mitochondrial function in liver and muscle[196]. Sirt4 reduces plasma FFA but, in turn, increases reactive oxygen species. In obese patients with NAFLD, the combination of FFA and oxidative stress products results in endothelial dysfunction and can be a coronary risk factor[197]. Oxidative stress is an important feature of the pathogenesis of NAFLD. As IGF-1 is known to have antioxidative effects and improve mitochondrial function, low IGF-1 levels may enhance oxidative stress and promote NAFLD[198,199].

Interventional studies regarding GH substitution are controversial. Some studies found an improvement after GH replacement[200], whereas others found only a reduction of abdominal and visceral fat without any impact on liver fat[201]. In one study the prevalence of NAFLD among patients with GHD was significantly higher than among controls (77% *vs* 12%, $P < 0.001$)[200]. After the introduction of GH replacement therapy, a reduction in the levels of liver enzymes and fibrosis markers (hyaluronic acid and type Ⅳ collagen) were noticed. Six months of GH-replacement therapy improved NASH and reduced oxidative stress[202]. GH replacement therapy also decreased serum levels of hsCRP and TNF-α, and drastically reversed NASH[202].

To our knowledge, there is data concerning NAFLD in acromegalic patients.

Hyperprolactinemia: Prolactin may be elevated in diverse conditions such as pituitary adenomas or by certain drugs. Hyperprolactinemia is seen in men with liver disease as well and is unrelated to the presence of gynecomastia[203]. Prolactin is not only a lactotroph hormone, but also regulates enzymes and transporters associated with glucose metabolism (stimulates insulin secretion) and lipid metabolism (suppresses lipid storage and adipokine release)[204-206]. Furthermore, adipose tissue produces prolactin in an autocrine and paracrine manner. Therefore, a potential role of prolactin in NAFLD may be evoked but has never been studied.

Bromocriptine, a dopamine agonist, has been linked to improvements in obesogenic behaviors, hepatic lipid accumulation, glucose tolerance and mitochondrial oxidative stress in rats and was therefore proposed as a therapy for NAFLD[207]. However, prolactin levels were not measured in the study.

Thyroid gland: hypothyroidism

Thyroid hormones play an important role in hepatic lipid metabolism, increasing hepatic lipogenesis and enhancing β-oxidation[208]. Increased fatty acid oxidation may produce reactive oxygen species, damaging hepatocytes[209]. Therefore, hypothyroidism is associated with reduced lipolysis and decreased liver uptake of FFA derived from triglycerides. Moreover, thyroid hormones modify hepatic fat accumulation, affecting adiponectin regulation. Hence, thyroid hormones could control the development of fibrosis through the modulation of adiponectin[209,210]. Increased leptin and FGF21 secretion may also play a role in this pathogenesis[209].

Thyroid hormones mediate their actions through thyroid hormone receptors. Thyroid receptor α

(THRα) is ubiquitously expressed and THRβ is mainly expressed in the liver, brain and kidney[211]. Rodent studies show that THRβ agonists diminish hepatic lipid accumulation[212]. Mice lacking THRα do not develop high-fat diet-induced hepatic steatosis and IR[213]. Moreover, hypothyroidism has been associated with disorders of glucose and insulin metabolism involving IR[214] which can influence the development of fatty liver disease. The relationship between thyroid dysfunction and NAFLD is controversial. Both diseases share common features such as metabolic syndrome, obesity, IR and the disturbance of lipid metabolism[209]. There is, however, no proven cause-effect relationship between the two conditions. In an Indian cohort, patients with NAFLD had higher thyroid stimulating hormone (TSH) levels and lower free thyroxine levels than control subjects[215]. Overt hypothyroidism has been associated with NAFLD[216,217] with a prevalence of 30.2% *vs* 19.5% in control subjects, even after adjusting for age, gender, BMI, diabetes and hypertension[218]. In a Chinese study, the prevalence of NAFLD increased in parallel to the degree of hypothyroidism: 29.9% for subclinical hypothyroidism and 36.3% for overt hypothyroidism. Each 1U/L increment of TSH was associated with a 20% increase in NAFLD prevalence, independently of classical risk factors[217]. These findings were confirmed by other studies[218,219]. Several studies demonstrated that an increased TSH level is an independent risk factor for NASH in patients with NAFLD[220]. In a recent cross-sectional study, compared to the low normal range (< 2.5 mIU/L), TSH levels within the upper normal range (2.5-4.5 mIU/L) were associated with a 40% increased risk for NAFLD after adjusting for age, gender, BMI, waist circumference, triglyceride levels, HDL cholesterol levels, hypertension, and diabetes[217]. However, in an Iranian cohort, there was no statistically significant difference in serum TSH, free T4 or free T3 levels between participants with or without NAFLD[221]. Moreover, a study by Mazo *et al*[222] did not show any statistically significant association between NASH and hypothyroidism. Nevertheless, a recent systematic review of 11 studies on this subject suggests that hypothyroidism is an independent risk factor for NAFLD. The prevalence of hypothyroidism ranged from 15.2% to 36.3% among patients with NAFLD/NASH[209]. Although this association has not been uniformly reported, further research is needed to confirm previous findings. However, it is unclear whether a low-normal thyroid function, but still within the euthyroid range, is related to NAFLD[223]. A cross-sectional study in euthyroid elderly Chinese individuals found that the prevalence of NAFLD is negatively correlated with serum free thyroxine[224]. An Italian retrospective study showed that serum gamma-glutamyltransferase and ALT concentrations increase steadily along with TSH categories, after adjusting for gender, age, lipids and fasting glucose concentrations[225]. It is important to determine whether hypothyroidism has an impact on NAFLD pathogenesis, as hypothyroidism is easily identifiable and treatable. Conversely, hepatic steatosis may influence thyroid function[226].

The use of thyromimetics, which are thyroid hormone analogs that either have selective effects on the liver or the heart, or bind selectively to TRβ rather than to TRα without cardiac side effects, are under consideration. Such compounds could be powerful new tools to address some of the largest medical problems in developed countries, *i.e.*, obesity and related disorders such as NAFLD[227]. Interestingly, thyroid hormones also exert non-genomic effects attributable to naturally occurring iodothyronines apart from T4 and T3[228]. Further studies are needed in this field.

To our knowledge, there is no association between hyperthyroidism and NAFLD.

Adrenal gland

Different pathologies can affect the adrenal gland, several of which appear to relate with NAFLD. One study on patients with adrenal incidentalomas found that there is no increased incidence of NAFLD in these patients[229].

Glucocorticoids - Cushing syndrome: Hypercortisolism shares metabolic features with metabolic syndrome like IR, dyslipidemia, hypertension, visceral obesity and hepatic steatosis. Cortisol is known to impair insulin sensitivity, directly by interfering with the insulin receptor pathway or indirectly by stimulating lipolysis and proteolysis, thereby increasing FFA and amino acid release. In addition, plasma glucose is increased due to stimulated gluconeogenesis[230].

However, patients with Cushing's syndrome have a low prevalence of hepatic steatosis[231], estimated at 20%[232]. It is hypothesized that there is a local increase of available glucocorticoids through the enzymatic activity of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1)[233]. Indeed, in obese individuals, there is increased regeneration of cortisone to cortisol mediated by increased activity of 11β-HSD1. This enzyme is expressed in the brain, adipose tissue and liver[234], and converts cortisone into active cortisol, which is able to promote metabolic changes[235]. However, not all studies have shown an increase of 11β-HSD1 activity in obese patients[236]. Another possible factor in NAFLD pathogenesis in Cushing syndrome is decreased clearance of cortisol through A-ring metabolism (5α- and 5β-reductase). Indeed, 5α-reductase type 1 deletion accelerates the development of hepatic steatosis[237]. In the early stages of NAFLD, only hepatic steatosis is observed. During this time, to protect the liver from cortisol exposure, 5α-reductase activity is increased, thus increasing cortisol clearance. Concomitantly, 11β-HSD1 activity is decreased, decreasing cortisol production,

which results in hypothalamic-pituitary-adrenal axis activation[238]. In the latter stages of NAFLD, especially in NASH, there is an increase in hepatic 11β-HSD1 expression[239], which increases intra-hepatic glucocorticoid levels. In addition, increased expression of glucocorticoid receptor α and decreased activity of 5α-reductase accentuate this mechanism, resulting in hepatic lipid accumulation. Therefore, progression of NAFLD is complex, with a switch from glucocorticoid inactivation to activation[240].

11β-HSD1 inhibitors are currently being developed to impair this phenomenon. Such an approach may be beneficial during the initial phase of steatosis but deleterious afterwards, notably by increasing inflammation[240].

Low levels of dehydroepiandrosterone/dehydroepiandrosterone sulphate: The effects of low levels of dehydroepiandrosterone (DHEA), an important and abundant steroid that influences oxidative stress, insulin sensitivity and expression of PPARα, is controversial. A positive relationship between histologically advanced NAFLD and low levels of dehydroepiandrosterone sulphate (DHEA-S) has been found, but the study was performed with two different groups, one in obese patients undergoing surgery and the other in suspected NAFLD patients[241]. Indeed, low serum levels of GH and DHEA are very common in patients with NASH and more advanced fibrosis[242]. Moreover, another group found high levels of DHEA-S in patients with NAFLD, but NAFLD wasn't histologically diagnosed[243]. Therefore, it remains unclear whether DHEA plays a role in NAFLD pathogenesis, or if this was an isolated finding.

Hyperaldosteronism: The Renin-Angiotensin-Aldosterone-System (RAAS) acts not only in the vascular system, but also in different organs such as the liver. Indeed, the angiotensin Ⅱ receptor 1 (AT1) and receptor 2 (AT2) are abundant in different tissues, and in the liver the former is expressed in hepatocytes, bile duct cells, HSC, KC, myofibroblasts, and vascular endothelial cells[244]. AT1 receptor activation by angiotensin Ⅱ induces HSC contraction and proliferation, causes oxidative stress, endothelial dysfunction, cell growth and inflammation[245]. The expression of AT2 in the liver has also been reported[246], with possible anti-fibrogenic effects.

An Italian cross-sectional pilot study found that in a selected population without other metabolic risk factors, patients with primary hyperaldosteronism and hypokalemia have a higher prevalence of NAFLD than normotensive controls[247]. Insulin sensitivity was lower in this group of patients, either impaired directly by aldosterone or indirectly by potassium loss[248]. Indeed, RAAS activation can increase IR. Angiotensin Ⅱ stimulates phosphorylation of serine residues in the insulin receptor β-subunit and the p85 regulatory subunit of PI3-kinase, thereby inhibiting the interactions between these two components of the insulin signaling pathway[249]. Activation of NADPH oxidase subsequently generates reactive oxygen species which modulate the production of pro-inflammatory cytokines such as TNF-α and IL-6, resulting in the impairment of insulin signaling[250]. There is a growing interest in using RAAS inhibitors to treat NAFLD. Indeed, blocking RAAS reduced fibrosis in an experimental model of hepatic fibrosis[251]. Telmisartan and valsartan improved transaminases levels and insulin sensitivity, and telmisartan also significantly decreased NASH activity score and fibrosis[252]. Despite encouraging results in animal studies, RAAS inhibitors do not show consistent efficacy in NAFLD patients. A recent study by Goh *et al*[253] demonstrated that in a hypertensive cohort with biopsy-proven NAFLD, patients treated with RAAS inhibitors had less advanced hepatic fibrosis, indicating a beneficial effect of this class of anti-hypertensive drugs. This effect remains controversial, however, as some studies did not observe any benefit of RAAS blockers on hepatic fibrosis[251,254].

Larger randomized clinical trials are needed to directly assess the effectiveness of angiotensin converting enzyme inhibitors and angiotensin Ⅱ receptor blockers in NAFLD.

TREATMENT

In endocrine disorders, the most appropriate course of action is first to treat the underlying disturbance. However, in the case of metabolic diseases such as diabetes or obesity, alternative approaches are needed.

Lifestyle is the first-line therapy[179,255]. A weight loss superior or equal to 7% improves histological disease activity[179]. Diet and exercise improve weight loss, steatosis and lobular inflammation[256]. Dietary composition is important, as reducing carbohydrate or fat intake can reduce intrahepatic lipid content[257]. It is important to emphasize that certain diets such as the low carbohydrate and high fat diet or diets rich in fatty acids or refined carbohydrates may exacerbate NAFLD[255]. Ketogenic diets impair fibroblast growth factor 21 (FGF21) signaling and enhance lipid accumulation in the liver, which may explain hepatic inflammation[258]. Dietary interventions can also modify gut microbiota, as already discussed. However, aggressive weight loss (> 1.6 kg/wk) removes lipids and fatty acids from visceral fat that can be taken up by the liver, exacerbating hepatic inflammation[259]. Some diets are more prone than others to NAFLD. Notably, diets high in saturated fatty acids, low carbohydrate diets, or diets rich in refined carbohydrates such as soft drinks, can exacerbate NAFLD[255,260], although this issue is controversial.

Insulin sensitizers

IR plays a pivotal role in NAFLD pathogenesis. Therefore, insulin sensitizers have been proposed as a

treatment. Metformin, a biguanide used for type 2 diabetes, decreases hepatic gluconeogenesis and lipogenesis[179,261]. However, there is no improvement in histology, and four randomized clinical trials have failed to demonstrate a significant beneficial impact of metformin on NAFLD progression[262]. Thiazolidinediones, PPARγ agonists used for type 2 diabetes, have effects on adipose tissue and reduce liver fat deposition. They interact with metabolic regulators such as adiponectin, AMPK, Foxo1 and peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α)[263]. In the "Pioglitazone *vs* Vitamin E *vs* Placebo for Treatment of Non-Diabetic Patients with Nonalcoholic Steatohepatitis" (PIVENS) trial[264], pioglitazone failed to meet the primary endpoint, *i.e.,* improvement of histologic features of NASH (fibrosis score), but improved ALT/AST levels, hepatic steatosis, lobular inflammation, insulin sensitivity, and steatohepatitis. Vitamin E improved steatohepatitis but not significantly. A pilot study reported that pioglitazone improved biochemical and histological features of NAFLD (steatosis, cell injury, inflammation, Mallory bodies, fibrosis), but there was no control group[265]. Recently, Pawlak *et al*[266] showed that the transrepression activity of PPARα may prevent progression of NASH to liver fibrosis. A meta-analysis of seven randomized clinical trials with post-treatment histology reported that thiazolidinediones improved histological activity (steatosis, hepatic ballooning, inflammation), plasma glucose and lipid levels, and reduced the risk of fibrosis progression[179]. However, the side effects (increased weight, raised fluid retention and heart failure[267], fractures, and bladder carcinoma) may limit the use of this class of drugs. Four randomized clinical trials with either pioglitazone or rosiglitazone confirmed the improvement of steatosis, ballooning and lobular inflammation, but did not address the long-term effects[262]. Finally, incretins, neuroendocrine hormones produced by the gastrointestinal tract in response to food, stimulate insulin release and decrease glucagon levels. Glucagon-like peptide-1 agonists and dipeptidyl peptidase-4 inhibitors are currently being studied, and transaminase levels have been shown to be reduced under these treatments[268].

Lipid-lowering drugs

Two types of lipid-lowering agents have been used in clinical studies: 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (statins) and ezetimibe. Statins have many effects, one of which is PPARγ agonism. Unfortunately, only a few pilot studies with small cohorts have used statins in monotherapy to evaluate hepatic histology in NAFLD[269,270]. Hyogo *et al*[270] found that atorvastatin improved transaminases levels and steatosis, but four patients had progression of fibrosis. In another study, statins improved hepatic steatosis and transaminases levels[271] but the impact on histology was not addressed[179]. Ezetimibe, an antagonist of Niemann-Pick C1-like protein, a key player in cholesterol absorption from the small intestine, may have an impact on NAFLD, but large-scale studies are needed to confirm this effect[272]. The combination of statins and ezetimibe, along with lifestyle changes, may represent a useful approach[273]. In summary, both classes of lipid-lowering drugs show promising results but need further investigation.

Anti-hypertensive drugs

Angiotensin Ⅱ receptor blockers inhibit hepatic inflammation and fibrosis *via* inhibition of fibroblast activity and prevention of HSC proliferation. A few studies have shown an improvement in liver histology and transaminases levels with the use of angiotensin Ⅱ receptor blockers[274]. Both telmisartan and valsartan were beneficial, but there was only one randomized clinical trial[252]. Details of this trial were discussed above.

Anti-oxidants and cytoprotective agents

Anti-oxidant and cytoprotective therapies have been evaluated for their effects on the inflammatory component of NAFLD. Vitamin E was studied in the PIVENS trial[264] and found to significantly improve hepatic steatosis but not fibrosis. However, the safety of large doses of vitamin E must be demonstrated, as it can increase IR and plasma triglyceride levels[179,275].

Betaine, a metabolite of choline which reduces oxidative stress, was tested but did not improve steatosis[276]. Ursodeoxycholic acid, a bile acid with antioxidant properties, failed to improve histological features[277,278]. As for pentoxyphylline, a TNF-α inhibitor, only one study showed an improvement in histology (steatosis and lobular inflammation, with only a trend for fibrosis) and transaminases levels[279].

Salsalate, a prodrug of salicylate with anti-inflammatory effects, was found to decrease steatosis and can therefore represent a new target drug if confirmed in larger studies[280].

TNF-α inhibitors like etanercept have been studied in patients with psoriasis. These inhibitors reduced transaminase and fasting insulin levels while exhibiting anti-inflammatory effects and improved insulin sensitivity.

Probiotics

Probiotics have been studied in a few trials, as previously discussed in the section on gut microbiota.

FGF21 analogs

FGF21 is an endocrine factor of the fibroblast growth factor family that improves insulin sensitivity in rodent models of IR. Administration of FGF21 decreased hepatic fat content and improved glucose homeostasis in mice[281,282]. Increased serum levels of FGF21 are found in patients with NAFLD, perhaps due to FGF21

resistance[283-286]. FGF21 analogs have been studied in humans and improve dyslipidemia, decrease body weight and fasting insulin plasma levels and increase adiponectin levels[287]. Several drugs are thought to regulate the FGF21 pathway, including resveratrol, a natural Sirtuin1 activator[288]. In diabetic rhesus monkeys, FGF21 administration improves insulin sensitivity and the lipid profile[289]. The potential beneficial effects of FGF21 in NAFLD patients warrant further investigation.

Gastric bypass

Surgical procedures such as bariatric interventions (notably gastric bypass) may lead to the resolution of liver steatosis. In one study of patients biopsied at the time of bariatric surgery and at follow-up, hepatic fat content was reduced in 65 out of 91 patients, whereas increases in the steatotic score were observed in only three patients[290]. Another study of 90 biopsied bariatric surgery patients showed that 16 patients (18%) had the same degree of steatosis, 25 (28%) had improved steatosis, and 49 (54%) had normal hepatic tissue in the second biopsy[291]. A recent French prospective study of 109 patients with morbid obesity and histologically-proven NASH showed that, one year after bariatric surgery, NASH had disappeared in 85% of the patients. The results were better in patients with mild NASH before surgery (94%) than severe NASH (70%), according to the Brunt scores. Histologically, steatosis decreased from 60% of the tissue before surgery to 10%, hepatocellular ballooning was reduced in 84.2% of samples, lobular inflammation was reduced in 67.1% of samples and fibrosis was reduced in 33.8% of the patients, according to the Metavir scores[292]. However, the guidelines indicate that it is premature to consider bariatric surgery as an option to treat NASH[293].

Orlistat, a lipase inhibitor, was tested in a pilot study of 10 obese patients, resulting in weight loss and improved aminotransferase levels, steatosis, and fibrosis[294].

CONCLUSION

It is important to note that NAFLD, the most common chronic liver disease in Western countries, is intimately entangled with various endocrine diseases, sharing the keystone physiopathological mechanism of IR. In the coming years, genetics will allow us to better understand the interrelationships between these different entities in order to better target treatments. Additional studies are needed to reveal the subtle links between common diseases like NAFLD and hypothyroidism, for example, and ensure their inter-dependence. Regarding treatment, we have seen that many drugs are useful not only for preventing the evolution of liver disease, but also against IR found in metabolic diseases. Prevention of metabolic syndrome is still important to prevent progression of NAFLD. Reciprocally, both gastroenterologists and endocrinologists should consider the relationship between NAFLD and endocrine diseases in everyday medical practice.

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TOPIC HIGHLIGHT

2015 Advances in Nonalcoholic Fatty Liver Disease

Liver fibrosis in non-alcoholic fatty liver disease - diagnostic challenge with prognostic significance

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease in the Western world, with a prevalence of 20%. In a subgroup of patients, inflammation, ballooning degeneration of hepatocytes and a varying degree of fibrosis may develop, a condition named non-alcoholic steatohepatitis. Advanced liver fibrosis (stage F3) and cirrhosis (stage F4) are histologic features that most accurately predict increased mortality in both liver-related and cardiovascular diseases. Patients with advanced fibrosis or cirrhosis are at risk for complications such as hepatocellular carcinoma and esophageal varices and should therefore be included in surveillance programs. However, liver disease and fibrosis are often unrecognized in patients with NAFLD, possibly leading to a delayed diagnosis of complications. The early diagnosis of advanced fibrosis in NAFLD is therefore crucial, and it can be accomplished using serum biomarkers (*e.g.*, the NAFLD Fibrosis Score, Fib-4 Index or BARD) or non-invasive imaging techniques (transient elastography or acoustic radiation force impulse imaging). The screening of risk groups, such as patients with obesity and/or type 2 diabetes mellitus, for NAFLD development with these non-invasive methods may detect advanced fibrosis at an early stage. Additionally, patients with a low risk for advanced fibrosis can be identified, and the need for liver biopsies can be minimized. This review focuses on the diagnostic challenge and prognostic impact of advanced liver fibrosis in NAFLD.

Key words: Non-alcoholic fatty liver disease; Fibrosis; Mortality; Biomarkers; Elastography

Core tip: Non-alcoholic fatty liver disease (NAFLD) has a prevalence of 20% in the Western world. A subgroup of NAFLD patients develops inflammation and fibrosis or cirrhosis. This condition, named non-alcoholic steatohepatitis, is associated with increased

mortality in liver-related and cardiovascular diseases. Advanced liver fibrosis is the histologic feature that most accurately predicts future morbidity; therefore, early detection of advanced fibrosis is crucial. Serum biomarkers, such as the NAFLD Fibrosis Score, Fib-4 Index or BARD, or non-invasive imaging techniques, such as transient elastography, may identify patients with a low risk for advanced fibrosis and minimize the need for liver biopsy.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease in the Western world. It has a global estimated median prevalence of 20%, ranging from 6.3% to 33% depending on the population, ethnicity, and assessment method for diagnosis[1,2]. Most patients have "simple steatosis" or non-alcoholic fatty liver (NAFL) without inflammation, tissue damage or fibrosis. However, in a subgroup of patients, non-alcoholic steatohepatitis (NASH), fibrosis and/or cirrhosis may develop. The prevalence of NASH in the general population is unknown, but it is estimated to be 3%-5%[1].

NAFLD is closely related to obesity, type 2 diabetes mellitus and dyslipidemia, with a prevalence ranging from 50% to 90% in these patient groups[2-4]. The current dogma implicates that NAFL is a stable disease with or without a very slow, histologic progression over time, whereas NASH may advance to fibrosis and cirrhosis[1,2,5-8]. However, several recent studies have challenged this view, demonstrating histological progression also in NAFL patients without histologic signs of NASH at baseline[9-12].

It is crucial for clinical management to obtain a prompt diagnosis of patients with advanced fibrosis because they carry an increased risk for developing complications such as hepatocellular carcinoma (HCC) or esophageal varices[5]. Consequently, patients with NAFLD who are diagnosed with advanced fibrosis or cirrhosis should be included in surveillance programs that utilize ultrasonography and endoscopy. In addition, recent data have noted that advanced fibrosis in NAFLD predicts not only liver-related mortality but also increased mortality due to cardiovascular events[13]. Therefore, patients with an increased risk for future complications must be identified sufficiently early to enable closer monitoring compared with those with a more benign course.

The prevalence of NAFLD is increasing, possibly due to the growing number of obese individuals in the Western world. In Ohio, United States, the number of patients with NAFLD among those listed for liver transplantation rose from 0% to 26% from 2000 to 2012. Similarly, the proportion of transplanted patients with NAFLD as the main diagnosis increased from 0% to 23.4% during the same time period[14].

Fibrogenesis in NAFLD is a critical process that affects clinical management. This review focuses on the natural course, diagnostic challenge and prognostic impact of advanced liver fibrosis on NAFLD.

HISTOPATHOLOGIC CLASSIFICATIONS OF NASH AND FIBROSIS

The current definition of NAFLD requires evidence of hepatic steatosis without signs of secondary hepatic fat accumulation due to alcohol consumption, steatogenic medication or hereditary disorders[5]. In the American Association for the Study of Liver Diseases (AASLD) Practice Guidelines, NAFL is defined as the presence of hepatic steatosis with no evidence of hepatocellular injury (ballooning of the hepatocytes), whereas NASH comprises the presence of hepatic steatosis plus inflammation with ballooning, with or without fibrosis[5].

In a pioneering publication from 1999, Matteoni *et al*[15] presented the first diagnostic criteria to categorize NAFLD into four different subtypes: NAFLD type 1 with fatty liver alone; type 2 with fatty liver plus lobular inflammation; type 3 with fatty liver plus ballooning degeneration; and type 4 with fat accumulation, ballooning degeneration and either Mallory-Denk bodies or fibrosis. In that study, fibrosis staging was not further evaluated. They demonstrated that cirrhosis developed in 21%-28% of patients whose liver biopsies displayed NAFLD type 3 or 4, whereas only 4% of patients with NAFLD type 1 and none of those with type 2 had cirrhosis development after a mean follow-up of 10 years. There was a trend for increased liver-related mortality in patients with subtypes 3 and 4 compared with subtypes 1 and 2. The subtypes 3 and 4 are those that we consider today to represent NASH[16].

There was a need for a more quantifiable grading and staging system, which was addressed by Brunt *et al*[17] during the same year. They developed a semi-quantitative system to grade NASH activity and stage NASH fibrosis. In their study, three grades of necro-inflammatory changes (mild, moderate and severe) were presented along with a staging score reflecting both the extent and location of fibrosis. Fibrosis Stage 1 encompassed perisinusoidal fibrosis, Stage 2 encompassed perisinusoidal with periportal fibrosis, Stage 3 included bridging fibrosis and Stage 4 included fully developed cirrhosis. The Brunt grading and staging system was based on the diagnosis of NASH depending on several histological features and not only one single attribute.

Table 1 Fibrosis staging in non-alcoholic fatty liver disease according to the Nonalcoholic Steatohepatitis Clinical Research Network Pathology Committee[18]

Perisinusoidal or periportal fibrosis	1
Mild perisinusoidal fibrosis (zone 3)	1A
Moderate perisinusoidal fibrosis (zone 3)	1B
Portal/periportal fibrosis	1C
Perisinusoidal and portal/periportal fibrosis	2
Bridging fibrosis	3
Cirrhosis	4

However, an increasing need for a more detailed scoring system did emerge. Such a system should enable assessment of the various histologic features during therapy and encompass the whole spectrum of NAFLD. Thus, the NASH Clinical Research Network (CRN) Pathology Committee performed a thorough univariate and multivariate analysis on the associations between the different histologic features observed in NASH and the diagnosis of NASH according to the Pathology Committee. The result was a scoring system of both NASH activity (Grade), and collagen deposition plus architectural remodeling (Stage). The grading system, the NASH Activity Score (NAS), was the unweighted sum of three histological components: steatosis (0-3), lobular inflammation (0-3) and ballooning degeneration (0-2). It ranged from 0 to 8. NAS includes the features of active injury that are potentially reversible[18]. Additionally, the fibrosis staging system of Brunt *et al*[17] was further developed. In the NASH CRN system, the fibrosis score for stage 1 was subdivided into delicate (1A) and dense (1B) peri-sinusoidal fibrosis, whereas stage 1C was defined as portal fibrosis without concomitant peri-sinusoidal fibrosis[18] (Table 1). The NASH CRN fibrosis staging system is one of the most validated systems currently available[19].

NAS has become widely accepted and used in clinical trials[20-22], and it is recommended as an endpoint in trials evaluating short-term treatments of NASH[19]. Thus, NAS has proven useful for comparative analyses and interventional studies but less beneficial as a diagnostic tool of NASH because neither fibrosis nor the location of lesions is included[23]. However, some authors still consider the numerical composite score of the NAS value to define whether NASH is present. However, in the original study by Kleiner *et al*[18], 16% of patients with a NAS $\geqslant$ 5 did not meet the diagnostic criteria for NASH. Thus, NAS cannot be considered as a substitute for the diagnosis of NASH[23]. Additionally, NAS was shown to be a poor predictor of fibrosis progression; therefore, it has also been questioned as a suitable endpoint for clinical studies[24].

Later, Younossi *et al*[25] evaluated various pathologic criteria for the diagnosis of NASH, comparing inter-observer agreement and the ability to predict liver-related mortality. They demonstrated that the original Matteoni criteria for NASH was a better predictor for liver-related mortality than both the Brunt criteria and NAS[25]. In their study, fibrosis scoring was simplified into four categories: (1) centrilobular/perisinusoidal; (2) centrilobular plus periportal; (3) bridging fibrosis; and (4) cirrhosis. Among individual features, fibrosis stages 3-4 (advanced fibrosis) showed the best independent association with liver-related mortality. These data indicate that fibrosis is a better predictor of liver-related mortality than NAS, which only grades steatosis and necro-inflammatory activity[25].

Recently, a new algorithm was developed by the Fatty Liver Inhibition of Progression Pathology Consortium based on a composite score evaluating Steatosis, Activity and Fibrosis (SAF score). Initially, this score was developed for classifying NAFLD in morbidly obese patients[26], but it has now been validated in a cohort of patients with NAFLD and metabolic syndrome[27]. In contrast to NAS, the SAF score separates steatosis from necro-inflammation, two features that may have distinct prognostic potential. The SAF scores steatosis (0-3), ballooning degeneration (0-2), lobular inflammation (0-2), and fibrosis (0-4). NASH is present when steatosis is present and when both features of activity (ballooning and lobular inflammation) display at least grade 1. Interestingly, independent from the classification of whether NASH is present, the overall histological severity of disease is scored separately as mild disease (A < 2, F < 2) or significant disease (A $\geqslant$ 2, F $\geqslant$ 2), also considering fibrosis staging. Therefore, NAFLD patients with less fat but still advanced fibrosis, and without necro-inflammation, would be classified as having "significant disease", even though they did not fulfill the criteria of NASH. Thus, the fibrosis component has an impact on the SAF score that may be relevant for long-term prognostication, although the association between the SAF score and long-term liver-related mortality has not yet been evaluated.

NATURAL COURSE OF FIBROSIS DEVELOPMENT IN NAFLD

Progression of liver fibrosis is observed in one-third of patients 4-5 years after the first liver biopsy. Variables associated with progression are obesity and body mass index (BMI)[28]. In a study of 106 patients with NAFLD, fibrosis stage progressed in 37%, remained stable in 34% and regressed in 29%. Diabetes and body mass index were associated with fibrosis progression[29]. In a meta-analysis comprising ten studies with 221 patients, 37.6% had progressive fibrosis over a mean follow-up time of 5.3 years. In this analysis, only age and inflammation in the initial biopsy were independent predictors of fibrosis progression[30]. Thus, approximately one-third of NAFLD patients progress in the fibrosis stage during a five-year follow-up, some of whom have a more rapid course.

For a long time, patients with simple hepatic steatosis without inflammation were considered to have a benign course with little progression, whereas progression to cirrhosis was observed only in patients with steatohepatitis[1,2,5-8,31,32]. However, this view has been modified in studies demonstrating that steatosis alone may progress to NASH with fibrosis[12].

In a study from Hong-Kong, paired liver biopsies were evaluated, and 23% of patients with simple steatosis developed NASH over a three-year period, whereas the regression of NASH was only observed in one patient[9]. Weight loss and reduction in waist circumference were associated with stable disease activity and non-progressive fibrosis.

In a study on 108 NAFLD patients who underwent serial liver biopsies with a median interval of 6.6 years, 42% had fibrosis progression. Diabetes was significantly associated with fibrosis development. There was no significant difference in the proportion exhibiting fibrosis progression between patients with NAFL or NASH at index biopsy (37% *vs* 43%)[11].

In a recent study, 25 patients with NAFL and 45 patients with NASH and/or advanced fibrosis were followed with repeat liver biopsy for an average of 3.7 years. Among the patients with NAFL, 16 patients (64%) developed NASH, eight of which had severe ballooning and six with bridging fibrosis. Mild lobular inflammation or any degree of fibrosis conveyed a higher risk of progression than simple steatosis alone. Older age and deterioration of metabolic risk factors were associated with a more rapid progression[33].

A recent meta-analysis evaluated 411 patients with biopsy-proven NAFLD from 11 cohort studies (150 patients with NAFL and 261 patients with NASH). In the whole cohort, 33.6% of patients had fibrosis progression. This result was also observed in patients with NAFL but at a slower pace. In those with NAFL, it took an average of 14.3 years to progress one stage in fibrosis score; however, in those with NASH, the time to progress with one stage was halved to 7.1 years[10].

Taken together, the data indicate that fibrosis progression is also observed in patients with NAFL, particularly in those with mild inflammatory changes, delicate fibrosis, older age or deterioration of metabolic risk factors. However, patients with NASH have a more rapid course, with a significant risk for liver-related mortality[6].

PROGNOSTICATION OF NAFLD

Several studies have evaluated the overall and disease-specific mortality in NAFLD. Liver disease is the third leading cause of death in NAFLD after cardiovascular disease and malignancy[34]. In a 28-year follow-up of 118 Swedish patients with NAFLD, there was a 69% increased risk of death compared with the total population, which was adjusted for sex, age, and calendar period. Those with simple steatosis had a 55% increased risk; however, in those with NASH, the risk was increased to 86%[35]. In another Swedish cohort study of 129 patients with biopsy-proven NAFLD with a mean follow-up of 13.7 years, survival and causes of death were compared with a matched reference population. Mortality was increased in patients with NASH but not in those with NAFL. The major causes of death were cardiovascular and liver-related events[31]. In a recent paper, these two cohorts were merged in a study comprising 229 patients with a mean follow-up of 26 years. In that study, advanced fibrosis (stage 3-4) was an independent predictor of overall and disease-specific mortality, whereas NAS > 4 was not associated with increased mortality[13].

These results indicate that fibrosis has a strong association to long-term outcome, and they are in line with previous studies. The original NASH criteria presented by Matteoni *et al*[15], which include fibrosis staging, shows a better association with liver-related mortality than both the NAS or Brunt criteria. When evaluating distinct pathologic features, advanced fibrosis shows the best independent association with liver-related mortality[25].

Interestingly, non-invasive biomarkers of advanced fibrosis can also predict mortality. Three-hundred two patients with NAFLD were sub-grouped as low-risk (60%) and intermediate-to-high risk individuals (40%), according to the non-invasive NAFLD fibrosis score (NFS). In a multivariate analysis, a higher NFS at baseline was significantly predictive of death[36]. In another retrospective, multicenter cohort study of 320 patients with biopsy-proven NAFLD, non-invasive scoring systems correlated with an increased risk for liver-related complications or death, and NFS had the best performance to identify patients at risk[37].

Non-invasive biomarkers of liver fibrosis were also tested in 2312 patients with type 2 diabetes and/or dyslipidemia, and the patients were followed prospectively for 5-15 years. Biomarkers indicative of advanced fibrosis were associated with overall mortality in a multivariate Cox model[38].

In studies from tertiary centers, selection bias leads to a high proportion of patients with advanced fibrosis or NASH. By contrast, population-based studies on NAFLD demonstrate a considerably smaller proportion of patients with advanced fibrosis. In the National Health and Nutrition Examination Survey conducted in 1988-1994, mortality data were followed-up through December 31, 2006. NAFLD was diagnosed on ultrasonography examination in 3792 individuals, comprising 34% of the total cohort, but the NAFLD fibrosis score indicative of advanced fibrosis (NFS > 0.676) was only observed in 3.2%, whereas 71.7% had NFS consistent with a lack of significant fibrosis (NFS < -1.455). After a median follow-up of 14.5 years, NAFLD in general was not associated with higher mortality. However, subjects with a high NFS indicative of advanced fibrosis had a 69% increase in mortality, mostly from cardiovascular events, and independent of other known risk factors[39].

The major causes of death in NAFL are cardiovascular disease and cancer[8]. In type 2 diabetes, the diagnosis of NAFLD is associated with an increased incidence of cardiovascular events, and this association was independent of other metabolic risk factors, suggesting that NAFLD by itself confers an increased risk for cardiovascular disease[40]. Interestingly, 86% of the diabetic NAFLD patients had normal liver enzymes[40]. The same authors also investigated carotid artery intima-media thickness (IMT) in patients with diabetes and NAFLD, and they found a strong association with the degree of hepatic steatosis, necroinflammation, and fibrosis. After adjustment for other potential confounders, the grade of NASH activity and stage of fibrosis independently predicted carotid IMT in a logistic regression analysis[41].

Taken together, the data indicate that advanced fibrosis is a strong predictor of increased overall and liver-related mortality in NAFLD and that NAFLD itself is an independent risk factor for cardiovascular disease.

NON-INVASIVE DIAGNOSIS OF ADVANCED FIBROSIS IN NAFLD

Clinical and laboratory variables (serum biomarkers)

Clinical predictors of advanced fibrosis in NAFLD are male sex, Caucasian ethnicity, diabetes mellitus, obesity and increased aspartate transaminase (AST) or alanine aminotransferase (ALT) levels[42,43]. However, there is a poor correlation between ALT levels and NASH, or the stage of fibrosis[44]. In a study of 222 patients with NAFLD, 23% had normal ALT. The proportion of patients with advanced fibrosis was similar among those with normal and elevated ALT[5].

AST is a better predictor for advanced fibrosis than ALT. In early studies on NAFLD, an AST/ALT ratio > 1 was found to be associated with advanced fibrosis[43]. Another test that includes AST is the AST: platelet ratio index (APRI)[45], with a negative predictive value of 94% to exclude advanced fibrosis (F3-4) in NAFLD. Another laboratory parameter related to fibrosis is serum ferritin. In a study of 628 patients with biopsy-proven NAFLD, elevated serum ferritin (> 1.5 × ULN) was associated with the diagnosis of NASH, high NAS, and development of advanced hepatic fibrosis[46].

For clinical decision-making with the purpose of identifying patients with an indication for liver biopsy, several composite scores have been explored. In 2007, the NAFLD fibrosis score (NFS), based on six routine clinical parameters, was developed and validated in > 700 patients with biopsy-proven NAFLD[47]. The parameters are age, BMI, the presence of diabetes or impaired fasting glucose, the AST/ALT ratio, platelet count and albumin. A score below -1.455 has a high negative predictive value to exclude advanced fibrosis (stage 3-4), whereas a score > 0.676 predicts advanced fibrosis. Only patients in the indeterminate range between these two values need to undergo liver biopsy, thus avoiding up to 75% of biopsies[47]. In a meta-analysis from 2010, the pooled AUROC, sensitivity and specificity of NFS for the detection of NASH with advanced fibrosis was 0.85 (0.80-0.93), 0.90 (0.82-0.99), and 0.97 (0.94-0.99)[6]. The NFS is endorsed by current American guidelines as a screening test to exclude low-risk individuals from further investigations[5].

Another simple score that was developed to exclude the presence of advanced fibrosis in patients with NAFLD is the BARD score. It is based on three variables combined in a weighted sum (body mass index $\geqslant$ 28 represents 1 point, the AST/ALT ratio $\geqslant$ 0.8 represents 2 points, and diabetes mellitus represents 1 point). A score of 2-4 had an odds ratio of 17 (confidence interval: 9.2-31.9) to determine advanced fibrosis and a negative predictive value of 96%.

The FIB-4 index was first developed for patients with hepatitis C and HIV but has been validated and compared with other non-invasive markers in a cohort of 541 NAFLD patients[48]. FIB-4 is based on patient age, AST, ALT, and platelet count. This index was superior to both NFS and BARD in this specific cohort. An FIB-4 index $\geqslant$ 2.67 had an 80% positive predictive value, and a value $\leqslant$ 1.30 had a 90% negative predictive value to diagnose advanced fibrosis. These results were also confirmed in a Japanese study[49].

Recently, a new non-invasive score, the non-invasive Koeln-Essen-index (NIKEI) based on age, AST, AST/ALT ratio, and total bilirubin, was compared with the FIB-4 index[50]. NIKEI had a slightly better AUROC of 0.968 than 0.929 for the FIB-4 index. The authors concluded that NIKEI can reliably exclude advanced fibrosis in subjects with NAFLD, particularly if used in conjunction with the FIB-4 index.

More complex scores include markers related to matrix turnover. Guha *et al*[51] developed the "Enhanced Liver fibrosis panel" (ELF), a panel of tissue inhibitor of matrix metalloproteinase 1 (TIMP 1), hyaluronic acid (HA), and aminoterminal peptide of pro-collagen Ⅲ (P3NP). The ELF has an area under the curve (AUC) of 0.90 for distinguishing severe fibrosis. The addition of more variables from the NAFLD Fibrosis Score (NFS) improved the diagnostic performance of the ELF, yielding an AUC of 0.98, but these results have to be confirmed in larger studies. The ELF has also been validated in pediatric patients with NAFLD[52].

Another composite score is the Hepascore, originally developed for chronic hepatitis C, which includes six variables (age, sex, α2- macroglobulin, hyaluronic acid, bilirubin, γ-glutamyltransferase)[53]. The Hepascore seems to be more accurate than the BARD and APRI but is similar to the FIB-4 score[53].

Proprietary panels have also been developed to evaluate fibrosis in NAFLD. First, the FibroMeter™ -NAFLD includes seven variables (age, body weight, ferritin, platelets, AST, ALT, and fasting glucose). Its AUROC to predict significant fibrosis (F2-4) was better

than that of the NAFLD fibrosis score, however with similar accuracy to predict cirrhosis[54]. Second, the FibroTest™, which is based on a combination of age, gender, bilirubin, γ-glutamyltransferase, apolipoprotein A1, haptoglobin, and α2-macroglobulin, has a performance similar to the FibroMeter™-NAFLD[55].

Adams *et al*[56] compared the performance of several scores and concluded that more complex scores (NFS, Fibrotest, Hepascore) perform better than simple ones (BARD). However, all scores based on biochemical parameters have modest accuracy for determining significant fibrosis (F2-4) with predictive values less than 90% in the majority of subjects, whereas the accuracy to exclude advanced fibrosis (F3-4) is better[56].

Which of these scores should be used in clinical practice? All of them have high negative predictive values to exclude advanced fibrosis[57-59]. Proprietary tests and more complex panels have the disadvantage of not being easily accessible in clinical everyday practice, whereas calculators for NFS, BARD score and the FIB-4 index are easily found on the Internet. In current American guidelines, the NFS is recommended[5,59], but some authors claim that BARD is easier to estimate than NFS[60], whereas other support FIB-4[61] or a combination of FIB-4 and BARD in a stepwise fashion[62].

Transient elastography

Transient elastography (TE; Fibroscan™) was first developed for the assessment of liver fibrosis in patients with chronic hepatitis C, in which it showed a good correlation with the METAVIR fibrosis stage[63]. The Fibroscan™ probe creates a low-frequency (50 Hz) elastic shear wave, which propagates through the liver tissue. The velocity of the shear-wave is measured and is directly related to tissue stiffness, which, in turn, is associated with the stage of fibrosis. Transient elastography is a quick and easy method, with a short procedure time and yielding immediate results. TE has been evaluated in patients with NAFLD in several studies[64-70]. In a meta-analysis, the pooled AUROC, sensitivity and specificity values of Fibroscan™ for the detection of NASH with advanced fibrosis were 0.94 (0.90-0.99), 0.94 (0.88-0.99) and 0.95 (0.89-0.99), respectively[6]. In another meta-analysis, transient elastography had an AUROC of 0.84-1.00 to exclude advanced fibrosis[71]. It had a high negative predictive value and a modest positive predictive value, indicating its usefulness as a screening test in the decision-making for liver biopsy. The cut-offs for excluding advanced fibrosis differ between various diagnoses. In NAFLD, liver biopsy may be considered in patients with a liver stiffness greater than 7.9 kPa using the M-probe (7.2 kPa with the XL-probe), a cut-off above which advanced fibrosis may occur[65].

The major pitfall for the use of transient elastography in NAFLD is the high failure rate due to invalid measurements in patients with high BMI and/or central obesity[67]. Failure rates lie approximately within 14%-17% using the standard (M-) probe[67,69,72] but can be improved to < 2% using the XL-probe[69]. Comparative studies on the M- and XL-probes show that the stiffness values with the XL-probe in general are 1.7 ± 2.3 kPa lower than those with the M-probe[66]. Therefore, separate cut-off values have been suggested for the XL-probe[57].

In a comprehensive review, Castera *et al*[57] suggest the sequential use of serum markers and elastography to predict the severity of fibrosis and help decision-making on whom to perform a liver biopsy for the staging of fibrosis (Figure 1). First, the use of the NAFLD fibrosis score (NFS) is suggested in patients with suspected NAFLD, as recommended by both the AASLD and European Association of the Study of the Liver guidelines[5,59]. Patients with intermediate NFS values (between -1.455 and 0.676) are further evaluated with transient elastography. A TE value < 7.9 kPa with the M-probe (< 7.2 kPa with the XL-probe) excludes advanced fibrosis with a negative predictive value of 89%-95%. These patients can be managed at primary care centers. However, a TE value > 9.6 kPa with the M-probe (> 9.3 kPa with the XL-probe) confirms advanced fibrosis with a positive predictive value of 72%[57], and patients should be screened with endoscopy for esophageal varices and ultrasonography for hepatocellular carcinoma. Thus, applying this algorithm, liver biopsies are only needed in patients with an NFS value between -1.455 and 0.676 and a Fibroscan value between 7.9-9.6 kPa with the M-probe (7.2-9.3 kPa with the XL-probe)[57].

Acoustic radiation force impulse imaging

Acoustic radiation force impulse imaging (ARFI) uses short-duration acoustic pulses that generate shear waves, which propagate through tissues and generate small tissue displacements[73]. ARFI is easily applied in ultrasonography machines that are commercially available and slightly modified. Two studies compared ARFI with transient elastography and found similar diagnostic performance between the two methods[74,75]. Cut-off values for advanced fibrosis in NAFLD have not yet been validated in larger studies.

Magnetic resonance elastography

In MR elastography (MRE), acoustic shear waves with frequencies between 40 and 120 Hz are generated by a pneumatic or electromechanical driver that is placed adjacent to the abdominal wall of the patient lying in supine position[76]. A modified phase-contrast MRI sequence is used to image the propagation of the shear wave in the region of interest of the liver. The technique can be used on conventional MRI systems with additional hardware and software. A study from 2011 suggests that MRE could detect advanced fibrosis in patients with NAFLD with a high accuracy[75]. In a

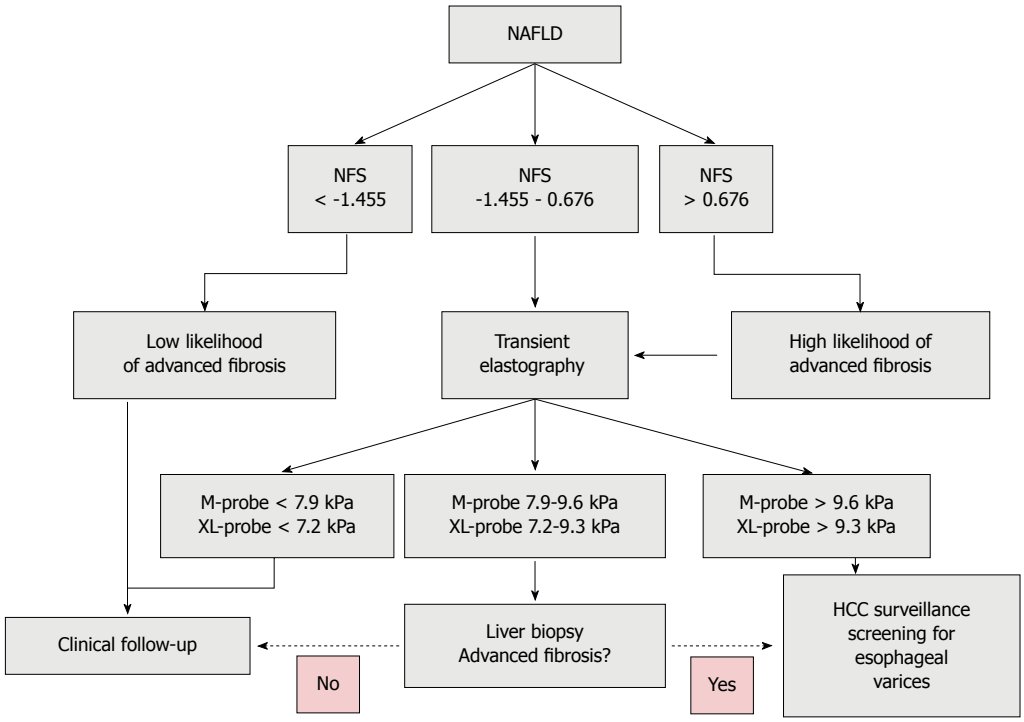

Figure 1 Proposed algorithm for the non-invasive diagnosis of advanced fibrosis in non-alcoholic fatty liver disease, as suggested by Castera *et al*[57]. NFS: NAFLD Fibrosis Score; NAFLD: Non-alcoholic fatty liver disease; HCC: Hepatocellular carcinoma.

recent prospective study of 102 patients with biopsy-proven NAFLD, the MRE had a high diagnostic accuracy for predicting advanced fibrosis (AUROC 0.957)[77]. The MRE technique is time-consuming and has a high cost; therefore, it has not yet been established in clinical practice.

SHOULD RISK GROUPS BE SCREENED FOR FIBROSIS?

One may argue that the treatment of NAFLD is the same regardless of whether fibrosis is diagnosed-*i.e.*, weight loss, increased physical activity and optimal glucose control if diabetes is present. Recent data demonstrate, however, that NAFLD patients with advanced fibrosis or cirrhosis have an increased risk for liver-related mortality, particularly the development of HCC. This risk is estimated to be > 2% per year if cirrhosis is present[78]. Only small HCC tumors found at an early stage have a potential for cure, if treated with liver transplantation, hepatic resection or local ablation. Patients with NAFLD and advanced fibrosis should therefore be evaluated if they are candidates for HCC surveillance with semiannual ultrasonography investigations.

Presently, HCC surveillance is largely defective in patients with NAFLD. In a recent study on the use of HCC surveillance in clinical practice, the diagnosis of NAFLD increases the risk of not receiving surveillance more than two-fold[79]. In more than one-third of HCC patients with NAFLD, surveillance is missed as a consequence of undiagnosed liver disease, compared with only 7.5% in patients with hepatitis C. Furthermore, in NAFLD, only 13% of HCCs are discovered by surveillance compared with 35% in hepatitis C. Even if HCC can be encountered in non-cirrhotic livers[80], the incidence increases with concurrent cirrhosis.

In a cohort of 1500 patients with hepatocellular carcinoma from Veterans Administration (VA) hospitals in the United States, NAFLD is the third most common risk factor for HCC and is observed in 8% of cases. Fifty-eight percent of NAFLD cases have underlying cirrhosis, and a lower proportion of these cases received treatment compared with HCV-associated HCC cases[81]. Thus, undiagnosed liver disease or unrecognized advanced fibrosis is common in NAFLD, leading to a high proportion of HCC patients who can only be offered palliative treatments[79].

One patient group in whom screening for NAFLD and advanced fibrosis would be feasible is type 2 diabetics. In a study of 1918 patients with diabetes, > 98% had reliable elastography measurements (1770 with the M probe and 114 with the XL probe). The proportion of patients with increased liver stiffness was 17.7%. Ninety-four patients underwent liver biopsy, and 50% of these had advanced fibrosis (F3-4)[82]. Thus, in this cohort of patients with diabetes type 2 and without any known liver disease, 2.3% were found to have undiagnosed advanced fibrosis or cirrhosis due to NAFLD.

CONCLUSION

NAFLD is the most common liver disease worldwide,

and with an increasing incidence. NAFLD is associated with an increased mortality in liver-related and cardiovascular events, the risk of which is highest in those with NASH and advanced fibrosis. The single histopathologic feature with the greatest impact on mortality is liver fibrosis, which can be divided into four stages (F1-4). One aim is to discover significant fibrosis (F2-4) in time to intensify treatment and delay further progression. If advanced fibrosis or cirrhosis (F3-4) has developed, there is an increased risk for hepatocellular carcinoma, and these patients should be considered for HCC surveillance. Screening tests to exclude advanced fibrosis comprise non-invasive serum biomarkers (NAFLD Fibrosis Score, BARD or FIB-4 index) or non-invasive imaging techniques based on liver stiffness measurements (transient elastography, ARFI or MRE). With these tests, patients without a risk of advanced fibrosis can be excluded, and the need for liver biopsies can be minimized. Strategies should also be developed to identify NAFLD patients with significant fibrosis among risk groups-*e.g.*, patients with type 2 diabetes and/or obesity.

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REVIEW

Genetic background in nonalcoholic fatty liver disease: A comprehensive review

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Abstract

In the Western world, nonalcoholic fatty liver disease (NAFLD) is considered as one of the most significant liver diseases of the twenty-first century. Its development is certainly driven by environmental factors, but it is also regulated by genetic background. The role of heritability has been widely demonstrated by several epidemiological, familial, and twin studies and case series, and likely reflects the wide inter-individual and inter-ethnic genetic variability in systemic metabolism and wound healing response processes. Consistent with this idea, genome-wide association studies have clearly identified Patatin-like phosholipase domain-containing 3 gene variant I148M as a major player in the development and progression of NAFLD. More recently, the transmembrane 6 superfamily member 2 E167K variant emerged as a relevant contributor in both NAFLD pathogenesis and cardiovascular outcomes. Furthermore, numerous case-control studies have been performed to elucidate the potential role of candidate genes in the pathogenesis and progression of fatty liver, although findings are sometimes contradictory. Accordingly, we performed a comprehensive literature search and review on the role of genetics in NAFLD. We emphasize the strengths and weaknesses of the available literature and outline the putative role of each genetic variant in influencing susceptibility and/or progression of the disease.

Key words: Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Genetics; Genome-wide association studies; Patatin-like phospholipase domain-containing 3; Transmembrane 6 superfamily member 2; Candidate gene studies

Core tip: Nonalcoholic fatty liver disease (NAFLD) is regarded as the most significant liver disease from the twenty-first century in the Western world. Although its development is surely driven by environmental factors, it is also regulated by genetic background. The role of heritability has been widely demonstrated by several studies, likely reflecting the diverse genetic variability in systemic metabolism and wound healing response processes. Accordingly, we performed a review of the literature on the role of genetics in NAFLD and

outlined here the putative role of each genetic variant in influencing susceptibility and/or progression of the disease.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) consists of a spectrum of disorders characterized predominantly by macrovesicular hepatic steatosis in absence of significant alcohol consumption. In this context, it is correct to discriminate between a condition of simple fatty liver, where the only histological finding is the presence of steatosis, and a state of nonalcoholic steatohepatitis (NASH), featured by hepatocellular injury and inflammation, with or without fibrosis[1]. NAFLD is regarded as the most relevant liver disease of the twenty-first century. Indeed, it has been estimated that NAFLD affects approximately 1 billion individuals worldwide[2]. It is the number one cause of altered aminotransferases in the Western world[3], where at least one third of the population is affected[4]. Importantly, a considerable proportion of NAFLD subjects (20%-30%) develop NASH, and this condition, as opposed to simple fatty liver, is a potentially progressive hepatic disorder that can lead to end-stage liver disease and hepatocellular carcinoma (HCC)[5]. In addition, several lines of evidence clearly demonstrated that all NAFLD/NASH patients are at high risk of cardiovascular diseases, type 2 diabetes (T2D), kidney failure, and colorectal cancer[6]. In this complex scenario, NAFLD development is surely driven by environmental factors - particularly dietary habits and a sedentary lifestyle - but it also requires a background of genetic susceptibility. Indeed, the real explanation for the wide inter-individual variability in the occurrence of NAFLD and progression to NASH - after correction for environmental factors - is provided by heritability. Much data has been accumulated over the years about the burden of heritability in NAFLD, as provided by epidemiological, familial, twin studies, and case series[7-10]. Furthermore, racial and ethnic differences have been reported in the prevalence of NAFLD, where it is most common in East Asian Indians, followed by Hispanics, Asians, Caucasians, and less frequently in African Americans[11-13]. In addition to differential exposure to metabolic risk factors, genetic variability in metabolism and wound healing response have surely influenced - at least in part - such differences. Not by chance, a great amount of evidence on the role of genetics in NAFLD/NASH has been produced during the last 10-15 years. Genetic studies can be divided into two categories: candidate gene studies and genome-wide association studies (GWAS). A GWAS is a hypothesis-free method for testing the association between all common variants in the human genome and polymorphic traits, such as diseases, drug response, and others. It is a powerful and statistically poorly biased method. On the other hand, candidate gene studies are generally derived from the results of previous genomic/proteomic and/or animal studies, where then a candidate gene is selected to investigate its putative role in the pathogenesis of a disease through a case-control single nucleotide polymorphism (SNP) study, with all potential methodological limits inherent to such type of study[14].

In this review, we have attempted to perform a comprehensive summary of the literature on the role of genetics in NAFLD/NASH, including the most recent evidence on genetic variants identified both by GWAS and candidate gene studies. Furthermore, we emphasize the strengths and weaknesses of the available literature for each variant, trying also to outline their putative role in NAFLD/NASH susceptibility and disease progression (Figure 1). Despite recent progress, several key issues remain to be addressed in the next years, particularly the details about the interaction between genetic background and acquired risk factors in disease pathogenesis and response to current treatments.

GENETIC VARIANTS AFFECTING NAFLD IDENTIFIED BY GWAS

Romeo *et al*[15] was the first to report that the rs738409 C>G SNP in the Patatin-like phospholipase domain-containing 3 (*PNPLA3*) gene, encoding the isoleucine to methionine variant at protein position 148 (I148M), was strongly associated with increased liver fat content. Since then, several other pieces of evidence have highlighted the role of PNPLA3 in the development and progression of NAFLD. Furthermore, other SNPs have been identified by GWAS (Table 1). Among them, transmembrane 6 superfamily member 2 (TM6SF2) E167K variant is currently emerging as another relevant contributor both for NAFLD pathogenesis and cardiovascular outcomes.

PNPLA3

The *PNPLA3* (also known as adiponutrin) gene encodes a transmembrane polypeptide chain exhibiting triglyceride hydrolase activity[16], which is highly expressed on the endoplasmic reticulum and lipid membranes of hepatocytes and adipose tissue[17]. PNPLA3 activity is regulated by glucose and insulin[18], mainly *via* pathways involving the sterol regulatory element binding protein-1c, as demonstrated both

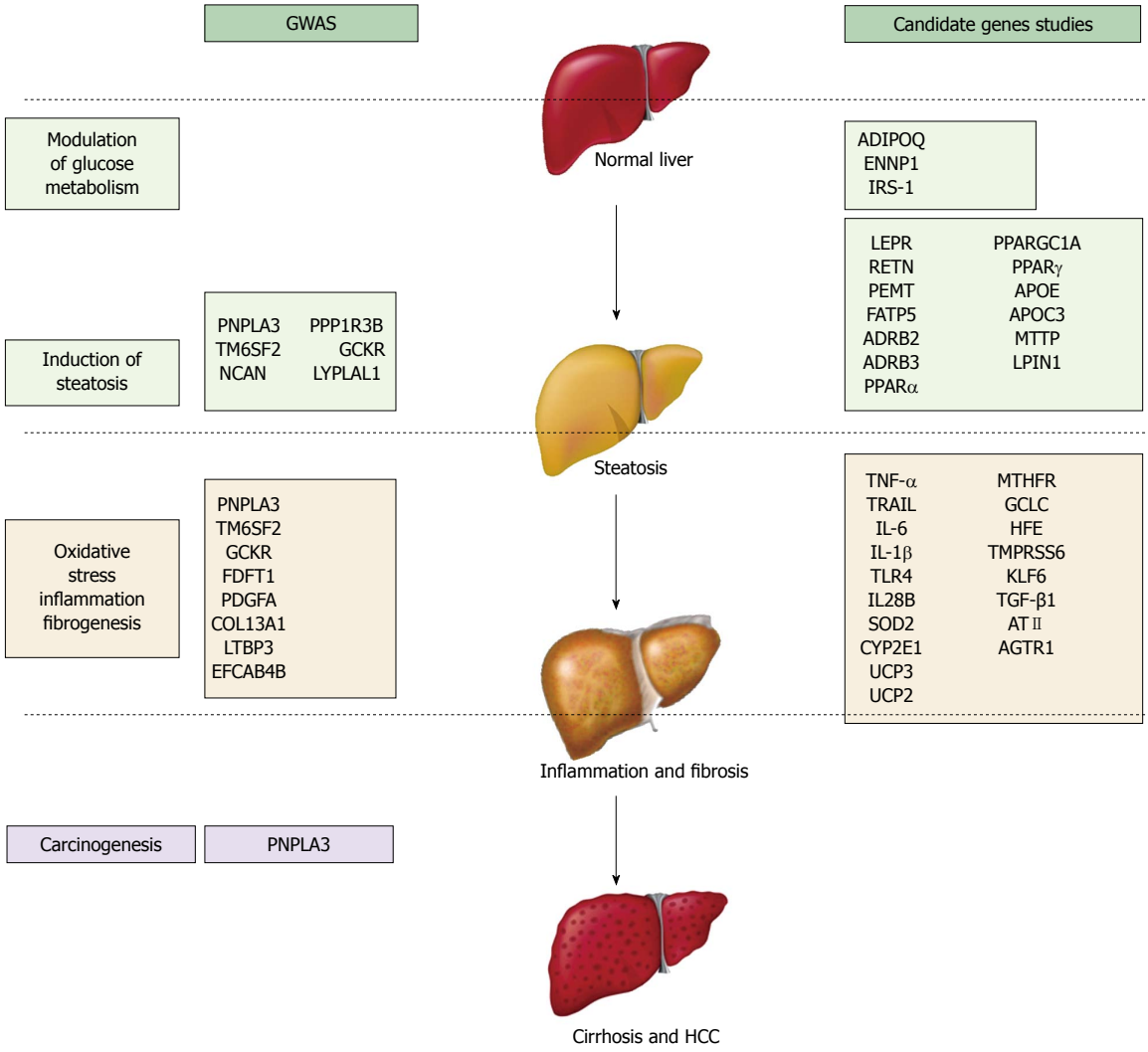

Figure 1 Hematic overview of the main genetic variants potentially involved in nonalcoholic fatty liver disease/nonalcoholic steatohepatitis susceptibility and progression. GWAS: Genome-wide association studies; HCC: Hepatocellular carcinoma.

in animal models and human hepatocytes[19]. The I148M variant - a SNP with a risk allele frequency of 21%-28% in European populations - impairs the phospholipase activity of the enzyme, thus reducing lipid catabolism, although it might also gain new functions[17] with a resulting increase in the synthesis of phosphatidic acid[20]. In addition, the PNPLA3 variant has been associated with a loss of retinyl-palmitate lipase activity in stellate cells[21]. Taken together, these data support a link between the PNPLA3 variant and the above reported wide spectrum of liver damage. As previously mentioned, the first report on the PNPLA3 I148M variant in NAFLD came from the GWAS by Romeo *et al*[15]. These authors identified the relationship between this SNP and liver fat content, and this association remained significant after adjusting for metabolic factors, ethanol use, and ancestry. Of great relevance, the link between PNPLA3 I148M variant and NAFLD is not confounded by the presence of metabolic syndrome (MS) and its features; indeed, even if some authors reported an interplay between insulin resistance (IR) and the variant[22,23], most studies did not find such association, as confirmed by a recent meta-analysis[24]. Interestingly, this independent association between the PNPLA3 I148M variant and NAFLD could be more relevant in women than in men, as highlighted by Speliotes *et al*[25] in a gender specific analysis performed on a histological NASH cohort. Beyond these gender differences, however, the PNPLA3 I148M variant could explain, at least in part, the variations in NAFLD prevalence across different multiple ethnicities. Indeed, the original report by Romeo *et al*[15] already found that the frequencies of the 148M allele matched the prevalence of NAFLD in the Dallas Heart Study[11], such that Hispanics had the highest frequency of the 148M allele (49%), followed by European Americans (23%) and African Americans (17%). These ethnic differences were subsequently confirmed by other investigators[26]. Over the last few years, several studies not only have further emphasized how the PNPLA3 I148M variant is associated robustly with liver fat content[27,28]

Table 1 Genetic variants involved in susceptibility and/or progression of nonalcoholic fatty liver disease identified by genome-wide association studies

Gene	SNP	Association with
PNPLA3, patatine-like phospholipase domain containing 3	rs738409	Steatosis
		NASH/necroinflammation
		Severity of fibrosis
		HCC development
TM6SF2, transmembrane 6 superfamily member 2	rs58542926	Steatosis
		NASH/necroinflammation
		Severity of fibrosis
		Reduced cardiovascular risk
NCAN, neurocan	rs2228603	Steatosis
PPP1R3B, protein phosphatase 1 regulatory subunit 3b	rs4240624	Steatosis
GCKR, glucokinase regulatory protein	rs780094	Steatosis
		Severity of fibrosis
LYPLAL1, lysophospholipase-like 1	rs12137855	Steatosis
FDFT1, farnesyl diphosphate farnesyl transferase 1	rs2645424	NAFLD activity score
PDGFA, platelet-derived growth factor alpha	rs343062	Severity of fibrosis
COL13A1, collagen type XIII alpha1	rs1227756	Lobular inflammation
LTBP3, latent transforming growth factor-beta-protein 3	rs6591182	Lobular inflammation
EFCAB4B, EF-hand calcium binding domain 4B	rs887304	Lobular inflammation

SNP: Single nucleotide polymorphism; NASH: Nonalcoholic steatohepatitis; NAFLD: Nonalcoholic fatty liver disease; HCC: Hepatocellular carcinoma.

but also revealed the link between the variant and the severity of liver injury, in terms of portal and lobular inflammation and Mallory-Denk bodies[29], presence of NASH, and severity of histological liver fibrosis[25,30] or liver stiffness measurement values[31]. This interplay between the PNPLA3 I148M variant and advanced fibrosis in patients with NASH has been further confirmed by a recent meta-analysis[32]. It is noteworthy that the role of PNPLA3 in NAFLD susceptibility and progression has been reported also in pediatric patients. In this line, the 148M allele was associated with higher liver fat content in Hispanic[33] and obese Taiwanese children[34], and with histological hallmarks of severity of liver injury - steatosis, hepatocellular ballooning and lobular inflammation, and presence of NASH and fibrosis - in Caucasian children and adolescents[35]. Interestingly, the PNPLA3 genotype seems to influence steatosis development also in chronic hepatitis C (CHC) patients, and it has been independently associated with the progression of CHC, including fibrosis, cirrhosis, and HCC occurrence[36,37]. Furthermore, it has been associated with susceptibility to steatosis in patients with chronic hepatitis B[38] and with cirrhosis and HCC development in patients with alcohol abuse[39,40]. Recently, the association between the *PNPLA3* variant I148M and the risk of HCC development has been robustly validated in patients with NAFLD[41,42], and it has been estimated that the homozygous carriers of the p.148M mutation carry a 12-fold increased HCC risk as compared to p.I148 homozygotes[43]. Considering all the aforementioned effects of PNPLA3 genotype on not only NAFLD, but also on alcoholic liver disease and CHC, some authors have proposed defining a novel clinical entity based on the presence of PNPLA3 risk allele - PNPLA3-associated steatohepatitis ("PASH") - *i.e.*, patients with fatty liver disease in whom PNPLA3 appears to be a major driver of disease progression in combination with ethanol consumption and Western diet[44]. Furthermore, PNPLA3 genotype has been evaluated as a possible modifier of NAFLD-associated systemic alterations. Our group recently examined the presence of carotid atherosclerosis in a Sicilian NAFLD cohort and its relation with several SNPs, including PNPLA3[45]. We found that the prevalence of carotid plaques and intima media thickness thickening was significantly higher in PNPLA3 GG compared to CC/CG genotype, particularly among patients under 50 years. This finding was also confirmed in a validation cohort from Northern Italy, where PNPLA3 GG genotype was independently associated with intima media thickness progression. Recently, Musso *et al*[46] associated the PNPLA I148M variant with the presence of chronic kidney disease, a well-known marker of a higher cardiovascular risk in NAFLD. Finally, a recent study by Sevastianova *et al*[23] evaluated whether weight loss was able to decrease liver fat in homozygous carriers of the G allele of PNPLA3; investigators found that liver fat content decreased significantly more in the 148MM group than in the 148Ⅱ after a short course of low carbohydrate diet, although 148Ⅱ and 148MM patients lost similar amounts of body weight. Overall, although the major role of PNPLA3 in susceptibility and progression of fatty liver has been widely elucidated, further research is needed to fully understand the role of PNPLA3 genotype on systemic alterations and treatment outcomes in patients with NAFLD/NASH.

TM6SF2

One of the most recently described and intriguing genetic factors in NAFLD scenario is the nonsynonymous variant rs58542926 (c.449 C>T) within a gene of mostly unknown functions called TM6SF2 at the 19p13.11 locus, which encodes an E167K

amino acid substitution. This variant is in strong linkage disequilibrium with other variants around the 19p13.11 locus that were previously reported by another GWAS (see further) to be risk factors for NAFLD[47], suggesting that the new and old signals could be the same, even if conditional analyses indicate that TM6SF2 rs58542926 may be the real causal variant underlying the association at this locus. The first evidence on this new SNP originated from three independent groups. Kozlitina *et al*[48] performed an exome-wide association study in a multiethnic, population-based cohort derived from the Dallas Heart Study, identifying the association between hepatic triglycerides content - evaluated by proton magnetic resonance spectroscopy - and the TM6SF2 variant rs58542926. In addition, the investigators highlighted the association between the TM6SF2 variant with higher serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels - as surrogate for NASH - and with reduced plasma levels of triglycerides and low-density lipoprotein (LDL)-cholesterol. Finally, they performed a functional analysis for the TM6SF2 in mouse models by silencing the gene *via* adeno-associated viral vectors. Silencing of the gene showed a 3-fold increase in hepatic triglycerides levels and a decrease in plasma levels of triglycerides, LDL- and high-density lipoprotein (HDL)-cholesterols and very low density lipoprotein (VLDL). Overall, their results demonstrated that the *TM6SF2* gene regulated hepatic triglyceride secretion and that the functional impairment of TM6SF2 promoted NAFLD. The second study conducted by Mahdessian *et al*[49] reported a positive correlation between hepatic TM6SF2 mRNA and plasma triglycerides levels and identified the subcellular localization and function of TM6SF2. Indeed, TM6SF2 was mainly localized in the endoplasmic reticulum and endoplasmic reticulum-Golgi intermediate compartment in human hepatoma cells. The TM6SF2 silencing in hepatoma cell lines reduced the expression of genes involved in the synthesis of triglycerides and the secretion of triglycerides-rich lipoprotein, demonstrating that TM6SF2 not only regulated hepatic lipoprotein secretion but also the hepatic synthesis of triglycerides. The third study reported by Liu *et al*[50] analyzed the relationship between the TM6SF2 rs58542926 SNP and the severity of liver disease in patients with biopsy-proven NAFLD. The authors found that the TM6SF2 rs58542926 SNP was associated with necroinflammation, ballooning, and advanced liver fibrosis. Taken together, these three studies provided evidence that the TM6SF2 variant was associated with the development of NAFLD/NASH *via* the deregulation of hepatic lipid metabolism. However, not all the authors reported unequivocal findings. Two studies, one from China[51] and one from South America[52], have been unable to replicate the relationship between TM6SF2 and NAFLD. This may be due ethnic differences in the frequency of carriage of the SNP and to the analysis of underpowered cohorts. Conversely, another study from China[53] confirmed, once again, the association between the *TM6SF2* 167K allele and NAFLD after adjusting for age, sex, body mass index, and presence of T2D. Thus, the reasons for such discrepancies have not yet been elucidated fully. The most interesting aspect about this variant, however, lies in its key role for the elucidation of the mechanistic basis of progressive NAFLD and for the development of a novel point of view on the association between NAFLD and cardiovascular disease. Consistent with this line, Dongiovanni *et al*[54] found that 188 (13%) out of 1201 subjects who underwent liver biopsy for suspected NASH were carriers of the E167K variant and that they had lower serum lipid levels than noncarriers, more severe steatosis, necroinflammation, ballooning, and fibrosis and were more likely to have NASH and advanced fibrosis after adjusting for metabolic factors and the I148M PNPLA3 risk variant. In addition, E167K carriers had lower risk of developing carotid plaque; in Swedish obese subjects assessed for cardiovascular outcomes, E167K carriers had higher ALT and lower lipid levels but also a lower incidence of cardiovascular events. Consequently, carriers of the TM6SF2 E167K variant seem to be more at risk for progressive NASH, but at the same time they could be protected against cardiovascular diseases. Furthermore, Musso *et al*[46] found that the TM6SF2 T allele was associated with higher eGFR and with a lower prevalence of albuminuria and chronic kidney disease - another known marker of an increased risk for cardiovascular disease in NAFLD. In other words, TM6SF2 may act as a switch gene able to disconnect the risk of NAFLD/NASH progression from cardiovascular risk.

Other genetic variants influencing NAFLD identified by GWAS

In 2011, Speliotes *et al*[47] aimed to discover additional genetic variants influencing NAFLD susceptibility using a genome wide analysis of hepatic steatosis assessed by computed tomography (CT) in large population based samples. First, authors confirmed the prominent role of rs738409 of PNPLA3 as the main genetic risk factor for NAFLD. In addition, they identified four other SNPs. These were localized in or near the genes neurocan (NCAN - rs2228603), protein phosphatase 1, regulatory (inhibitor) subunit 3B (PPP1R3B - rs4240624), glucokinase regulator (GCKR - rs780094), and lysophospholipase-like 1 (LYPLAL1 - rs12137855). NCAN, GCKR, and LYPLAL1, together with PNPLA3, were associated with both increasing CT hepatic steatosis and histological NAFLD, whereas PPP1R3B was associated with CT-assessed steatosis but not histological NAFLD. NCAN is involved in mechanisms of cell adhesion and in lipoprotein metabolism, and its locus was subsequently casually related to the TM6SF2 minor allele (see above). LYPLAL1 likely exerts a complementary function to the PNPLA3

protein in trigliceride catabolism. The protein product of GCKR has been proposed to interfere with glucose and lipid homeostasis *via* the interaction with hepatic glucokinase and the consequent increased activity of the enzyme[55], ultimately raising the hepatic glycolytic flux, *de novo* lipogenesis, and triglyceride levels[56]. Several genetic association studies have confirmed the connection between GCKR rs780094 and NAFLD[57-60], including progression of the disease and fibrosis[61]. These findings were further confirmed by a recent meta-analysis[62] that demonstrated a similar effect size of such association in both Asian and non-Asian populations.

Finally, Chalasani *et al*[63] reported another GWAS in 2010, identifying other variants conferring susceptibility to occurrence of NAFLD and disease progression. On a cohort of patients with biopsy-proven NAFLD, investigators demonstrated an association between severity of histological NAFLD activity score and SNP rs2645424 in the gene encoding farnesyl diphosphate farnesyl transferase 1 - an enzyme involved in cholesterol biosynthesis. Strangely, they did not identify PNPLA3 as a risk factor. However, other associations were reported, including SNP rs343062 on chromosome 7 (near platelet-derived growth factor alpha gene) with the degree of fibrosis; SNP rs1227756 on chromosome 10 in the collagen type XIII alpha1 (*COL13A1*) gene, rs6591182 on chromosome 11 (near latent transforming growth factor-beta-protein 3 gene), and rs887304 on chromosome 12 in EF-hand calcium binding domain 4B (*EFCAB4B*) gene with lobular inflammation; and SNP rs2499604 on chromosome 1, rs6487679 on chromosome 12, rs1421201 on chromosome 18, and rs2710833 on chromosome 4 with serum levels of ALT. However, all of them require extensive validation in larger cohorts.

POTENTIAL GENETIC FACTORS INFLUENCING NAFLD/NASH IDENTIFIED BY CANDIDATE GENES STUDIES

Several genes have been identified as potential candidates in the pathogenesis and progression of fatty liver. In order to give a schematic overview, we roughly divided all candidate genes into two categories: genes influencing glucidic or lipid metabolism - directly or indirectly involved in fatty liver development (Table 2) - and genes involved in mechanisms of liver injury (Table 3).

Genes influencing glucidic or lipidic metabolism with a potential role in NAFLD pathogenesis

Ectonucleotide pyrophosphatase/phosphodiesterase1 or plasma cell antigen-1 and insulin receptor substrate 1: Insulin resistance - the hallmark of NAFLD pathophysiology - is strongly related to disease progression. Not by chance, SNPs of genes included in the hepatic insulin signalling pathway have consistently been reported to influence IR and to be potential causes of hepatic injury[64]. Among them, the ectonucleotide pyrophosphatase/phosphodiesterase1 (ENPP1)/plasma cell antigen-1 Lys121Gln SNP enhances the interaction between the ENPP1 membrane glycoprotein and the insulin receptor, resulting in inhibition of insulin receptor activity. This SNP has been associated with an increased risk of T2D[65]. Furthermore, the loss-of-function Gly972Arg SNP of IRS-1 - part of the machinery involved in the insulin signaling pathway - decreases activity of IRS-1, thereby inhibiting insulin receptor autophosphorylation and activity[66] and thus increasing the risk of IR and T2D[67]. Dongiovanni *et al*[68] analyzed the role of these two SNPs in influencing liver damage in 702 patients with biopsy-proven NAFLD from Italy and the United Kingdom, finding that both were independently associated with a marked reduction of insulin signaling activity and with increased the severity of liver fibrosis. Interestingly, the effect of the ENPP1 and IRS-1 SNPs on the severity of liver fibrosis was independent of ethnic background, as it was observed in patients from both Italy and the United Kingdom, thus emphasizing how hepatic IR has a causal role in the progression of liver damage in NASH.

Adiponectin: Adiponectin is a relevant adipocytokine associated with IR and T2D[69]. Several papers have demonstrated a significant decrease in the serum levels of adiponectin in NASH patients[70] and a reduced expression of its receptor in livers with NASH compared to those with simple steatosis[71]. Furthermore, adiponectin has been associated with liver fibrosis and inflammation[72,73], suggesting that it might be directly or indirectly involved in NASH pathogenesis. Variants in adiponectin (ADIPOQ) - the gene encoding adiponectin - have been investigated in order to find potential associations with NAFLD and its severity. Musso *et al*[74] showed that the at-risk ADIPOQ SNPs 45TT and 276GT were significantly more prevalent in NAFLD than in the general population and that they were associated with the severity of liver disease and with an atherogenic postprandial lipoprotein profile in NASH, independent of fasting adipokine and lipid levels. Consistent with this line, a Japanese study highlighted how such SNPs were associated with IR and progression of liver fibrosis in NAFLD Japanese patients[75]. However, these findings were not replicated in other cohorts. Although hypoadiponectinemia and IR were observed also in Chinese NAFLD patients, the 45TT and 276GT SNPs were not directly associated with NAFLD, even if they might have indirect effects on systemic metabolism and/or NAFLD pathogenesis by influencing serum ALT, body mass index, IR, and plasma adiponectin concentration[76]. It is possible that ethnic differences could explain the discrepancies among these studies.

Table 2 Genes influencing glucidic or lipid metabolism with a potential role in nonalcoholic fatty liver disease pathogenesis evaluated by candidate gene studies

Gene	Functions of encoded protein	SNP
ENPP1, ectonucleotide pyrophosphatase/ phosphodiesterase1 or PC-1	Interaction with the insulin receptor with consequent reduction of insulin receptor activity	rs1044498
IRS-1, insulin receptor substrate 1	Part of the machinery involved in insulin pathway as transductor of insulin receptor signaling	rs1801278
ADIPOQ, adiponectin	Relevant adipocytokine associated with insulin resistance, type 2 diabetes, and NAFLD pathogenesis	rs2241766 rs1501299
LEPR, leptin receptor	Receptor of leptin, a hormone synthesized by adipocytes that regulates food intake, insulin action, thermogenesis, and immune system	rs62589000 rs6700986 rs1137100 rs1137101 rs8179183
RETN, Resistin	Adipocytokine involved in lipid metabolism, hepatic insulin resistance, inflammatory cascade reactions, and fibrogenesis	rs3745367
PEMT, phosphatidylethanolamine N-methyltransferase	Enzyme involved in the *de novo* synthesis of phosphatidylcholine in the liver, a biochemical pathway essential for VLDL formation	rs7946
FATP5, Fatty Acid Transport Protein 5	Transporter involved in the hepatic uptake of fatty acids	rs56225452
ADRB2 and *ADRB3*, β-adrenergic receptor 2 and 3	β-adrenergic receptors, with several functions including regulation of basal metabolism and induction of lipolysis	rs4994 rs1042714 rs2053044 rs11168070 rs11959427 rs1042711
PPARα, peroxisome proliferative activated receptor α	Transcription factor whose activation improves steatosis, inflammation, and fibrosis in pre-clinical models of NAFLD	rs1800206
PPARGC1A, peroxisome proliferator-activated receptor γ coactivator 1-α	PGC-1α, involved in mitochondrial functions, oxidative stress, gluconeogenesis, and lipogenesis	rs8192678 rs2290602
PPARγ, peroxisome proliferative activated receptor γ	Transcription factor whose activation improves IR, restores adipose tissue insulin sensitivity, and decreases fatty free acids flux to the liver	rs1801282
APOE, apolipoprotein E	Mediator of remnant lipoprotein binding to LDL receptors to favor the clearance of chylomicrons and VLDL	N/A
APOC3, apolipoprotein C-Ⅲ	A constituent of plasma VLDL, chylomicrons, and HDL-C that inhibits lipoprotein lipase and triglycerides clearance	rs2854116 rs2854117
MTTP, microsomal triglyceride transfer protein	Transfer protein involved in apoB-lipoprotein assembly	rs1800591 rs1800804 rs1057613 rs3805335
LPIN1, lipin 1	Phosphatase specifically involved in metabolic pathways between adipose tissue and liver	rs13412852

SNP: Single nucleotide polymorphism; NAFLD: Nonalcoholic fatty liver disease; HDL-C: High-density lipoprotein-cholesterols; VLDL: Very low density lipoprotein; LDL: Low density lipoprotein.

Leptin receptor: Leptin is a hormone synthesized by adipocytes that regulates food intake, insulin action, thermogenesis, and the immune system[77]. Several studies[78,79] have demonstrated the association between serum leptin levels and risk of NASH, although results have been sometimes conflicting[80]. Accordingly, the leptin receptor has been investigated due to its potential relevance in the modulation of leptin sensitivity: common variants in the human leptin receptor (*LEPR*) gene have been related with obesity and lipid metabolism[81], IR and T2D[82], and NAFLD[83-86]. The LEPR G3057A variant has been associated with the risk of NAFLD in Chinese diabetic patients[83], whereas Swellam *et al*[84] showed that NAFLD occurrence was associated with another SNP in LEPR - rs6700986 - in an Egyptian cohort. Furthermore, Zain *et al*[85] investigated the relationship between polymorphisms in LEPR and NAFLD across different Asiatic ethnic groups (Malayan, Indian, and Chinese). Two SNPs (LEPR rs1137100 and rs1137101) were associated with susceptibility to NAFLD and NASH; and, intriguingly, analysis of gene-gene interaction showed a potential interplay between the *LEPR* and *PNPLA3* genes. Finally, Lys656Asn SNP of LEPR was associated with metabolic factors - namely IR, obesity parameters, and glucose levels - in patients with NAFLD[86]. Thus, LEPR variants may be involved in the occurrence and progression of NAFLD by influencing insulin sensitivity and/or lipid metabolism, even if further evidence should be provided to reinforce such observations.

Resistin: Resistin (RETN) is an adipokine with relevant metabolic actions and a potential role in NAFLD pathogenesis. Indeed, murine models showed that RETN is able to modulate lipid metabolism and hepatic IR[87,88] and may also participate in inflammatory cascade reactions known to be involved in NASH development[89] and in processes of fibrogenesis[90]. Many SNPs of *RETN* gene have been investigated as

Table 3 Genes potentially involved in mechanisms of liver injury in nonalcoholic fatty liver disease evaluated by candidate gene studies

Gene	Functions of encoded protein	SNP
TNF-α, tumor necrosis factor-α	Proinflammatory cytokine also involved in the regulation of insulin resistance, release of free fatty acids, and apoptosis in hepatocytes	rs1800629 rs361525 rs1799964 rs1800630
TRAIL, TNF-related apoptosis -inducing ligand	Protein functioning as a ligand that induces celluar apoptosis	rs6763816 rs4491934
IL-6, interleukin-6	Proinflammatory cytokine produced by adipocytes, hepatocytes, and immune cells also involved in the modulation of insulin resistance	rs1800795
IL-1β, interleukin-1β	Member of IL-1 family cytokine, mainly produced by adipose tissue	rs16944
TLR4, toll-like receptor 4	Receptor involved in the interaction with bacterial endotoxins capable to favor hepatic injury and a proinflammatory systemic status	rs4986790
IL28B, interleukin-28B	Cytokine belonging to the type Ⅲ Interferon family	rs12979860
SOD2, superoxide dismutase 2	Manganese-dependent mitochondrial enzyme involved in protection from cellular injury induced by superoxide radicals	rs4880
CYP2E1, cytochrome P450 2E1	Part of the cytochrome P450 complex	rs2031920
UCP3, uncoupling protein 3	Mitochondrial anion carrier involved in the metabolism of superoxide radicals and in the modulation of lipid homeostasis	rs1800849 rs11235972
UCP2, uncoupling protein 2	Similar to uncoupling protein 3	rs695366
MTHFR, methylenetetrahydrofolate reductase	Enzyme involved in the methylation of homocysteine to methionine	rs1801133 rs1801131
GCLC, Glutamate-cysteine ligase catalytic subunit	Limiting enzyme in the formation of glutathione, a relevant endogen antioxidant	rs17883901
HFE, hemochromatosis	Crucial protein for the regulation of iron homeostasis *via* the modulation of the expression of hepcidin	rs1800562 rs1799945
TMPRSS6, trans-membrane protease serine 6	Matriptase-2, which cleaves the membrane-bound hemojuvelin, the co-receptor required for hepcidin expression in the liver	rs855791
KLF6, kruppel-like factor 6	One of the Kruppel-like factors, a family of transcriptional factors that regulate cellular proliferation, differentiation, and apoptosis	rs3750861
TGF-β1, transforming growth factor β1	In the liver, a promoter of hepatic fibrosis *via* the activation of hepatic stellate cells	rs1800471
ATⅡ, angiotensin Ⅱ	Part of the renin-angiotensin system, also advocated as an inducer of TGF-β1 production and accumulation of extracellular matrix in the liver	rs699
AGTR1, Angiotensin Ⅱ Type 1 Receptor	Type 1 Receptor of Angiotensin Ⅱ	rs3772622 rs3772633 rs2276736 rs3772630 rs3772627

SNP: Single nucleotide polymorphism; TGF: Transforming growth factor.

potential risk factors for MS and its components[91]. A Chinese study[92] investigated the role of the RETN intronic +299G/A SNP in a NAFLD setting and found that patients with both T2D and NAFLD had the highest plasma RETN levels compared with diabetic patients without evidence of NAFLD and with controls. Furthermore, the AA genotype at the +299 site of the *RTEN* gene was found to be an independent risk factor for the development of NAFLD in T2D patients at multivariate analysis. However, further studies are needed to confirm this simple association.

Phosphatidylethanolamine N-methyltransferase: Phosphatidylethanolamine N-methyltransferase (PEMT) is a relevant enzyme involved in the *de novo* synthesis of phosphatidylcholine in the liver[93], a biochemical pathway essential for VLDL formation. Thus, PEMP is involved in the flux of lipid between the liver and plasma, where lack of phosphatidylcholine caused severe steatosis in mice models[94]. A higher frequency of a nonsynonymous sequence variation (V175M) in the *PEMT* gene, which results in a loss-of-function in the encoded protein, was reported in patients with biopsy-proven NAFLD compared with subjects with normal hepatic triglyceride content assessed by magnetic resonance or by liver biopsy[95]. Similarly, Dong *et al*[96] found that the occurrence of the V175M variant allele was significantly more frequent in 107 Japanese patients with biopsy-proven NASH than in 150 healthy controls. Conversely, Jun *et al*[97] did not find any difference in PEMT genotype frequency between NAFLD patients and controls, and Romeo *et al*[98] demonstrated a lack of any association between the V175M allele and hepatic triglyceride content - assessed by proton magnetic resonance spectroscopy - in their cohort derived from the Dallas Heart Study, a population-based sample from Dallas, Texas[99]. Overall, the available evidence is not enough to firmly consider PEMT as a relevant genetic factor for NAFLD susceptibility and more studies are needed in this setting.

Fatty acid transport proteins: Fatty acid transport proteins (FATPs) are critically involved in the uptake of fatty acids[100], and two different FATP isoforms are expressed in the liver, namely FATP2 and FATP5[101].

Mice models have emphasized the role of FATP5 in increasing the hepatic uptake and trafficking of fatty acids, so that gain-of-function polymorphisms may result in increased steatosis[102]. Auinger *et al*[103] investigated the consequences of the rs56225452 FATP5 promoter polymorphism on lipid and glucose metabolism and on features of MS in a cohort derived from the Metabolic Intervention Cohort Kiel - a prospective population-based cohort study of the town of Kiel, in Germany, on natural incidence of the MS[104] - and subjects with histologically proven NAFLD. Triglycerides, ALT, and postprandial insulin levels were higher in subjects with the A allele compared with GG homozygotes in the Metabolic Intervention Cohort Kiel cohort, whereas in NAFLD patients, the A allele was associated with higher ALT only. However, the impact of body mass index on the severity of steatosis differed according to FATP5 promoter SNP, suggesting that this polymorphism may be associated with MS and - probably indirectly - with liver damage in NAFLD. Additional independent studies are needed to fully clarify this interesting, even if still unclear, association.

β-adrenergic receptors: β-adrenergic receptors (ADRB) play an important role in regulating basal metabolism, mostly by stimulating lipid mobilization through lipolysis. Several polymorphisms have been detected in *ADRB* genes that influence IR, hypertriglyceridemia, and features of MS[105-108]. These polymorphisms were evaluated in NAFLD settings, although with conflicting results. A Japanese study involving 63 patients with biopsy-proven NASH analyzed a W64R codon substitution in *ADRB3* gene: the R allele frequency in patients with NASH was significantly higher compared with controls[109]. Other authors examined two nonsynonymous polymorphisms involving the *ADRB2* gene (*Gln27Glu* and *Arg16Gly*): no significant association with fatty liver was observed for the Arg16Gly allele, whereas the Gln27Glu heterozygotes showed a higher prevalence of fatty liver compared with those without the mutation at univariate analysis, even if this association was not confirmed at multivariate analysis[106]. Loomba *et al*[110] have published the most relevant study on ADRB2 in 2010. The authors evaluated whether common variants at *ADRB2* gene in twins were associated with plasma γGT levels - a well-known significant predictor of the MS as well as NAFLD[111,112]. Interestingly, five SNPs in ADRB2 were associated with levels of γGT, and ADRB2 haplotypes displayed pleiotropic effects on γGT and triglyceride levels, suggesting that adrenergic pathways may act as a link between genetic susceptibility to NAFLD and MS.

Peroxisome proliferative activated receptor α, peroxisome proliferative activated receptor γ, and peroxisome proliferator-activated receptor γ coactivator 1-α: Peroxisome proliferative activated receptor (PPAR) α is a transcription factor belonging, together with PPARγ and PPARβ/δ, to the NR1C nuclear receptor subfamily. PPARα activation improves steatosis, inflammation, and fibrosis in pre-clinical models of NAFLD[113], whereas PPARγ improves IR and has been reported to restore adipose tissue insulin sensitivity and decrease fatty free acids flux to the liver[114]. Regarding PPARα SNPs and NAFLD, a Chinese study evaluated the frequency of the val227ala variant on patients with NAFLD compared with control subjects[115]. As the distribution of PPARα val227ala polymorphism was significantly different between the two groups, the authors hypothesized that the Val227 isoform - the one predominant in NAFLD subjects - has lower activity than the Ala227 isoform, thus resulting in a reduced lipid catabolism and an increased risk for NAFLD. Another PPARα variant examined in a setting of NAFLD is the loss-of-function Leu162Val. Dongiovanni *et al*[116] did not find any association between this SNP and the risk of NAFLD occurrence and histological severity, although it was independently related to IR. The same study also assessed the Pro12Ala loss-of-function SNP in *PPARγ2* gene. Even if this polymorphism had been identified as an important mediator for the development of obesity, IR, and T2D[117], no significant association with NAFLD susceptibility and severity was found. Importantly, this SNP was not even associated with IR in this cohort. Similar conclusions were argued by a recent meta-analysis[118] including 1697 cases and 2427 controls derived from eight studies[116,119-125]. No clear evidence of an association between the Pro12Ala polymorphism and susceptibility to NAFLD emerged. The protein PGC-1a is encoded by the peroxisome proliferator-activated receptor γ coactivator 1-α (*PPARGC1A*) gene and regulates mitochondrial functions, oxidative stress, gluconeogenesis, and lipogenesis[126]. The Gly482Ser SNP in *PPARGC1A* gene has been repeatedly associated with T2D, hypertension, and obesity in clinical studies[127-129] and also with an impaired capability of PGC-1α to decrease fat deposition in cultured hepatocytes[130]. In this line, it was also associated with the development of NAFLD in Taiwanese obese children after controlling for body mass index, sex, and PNPLA3 genotype[131]. Yoneda *et al*[132] examined 15 SNPs in PPARGC1A in the Japanese population; they found that rs2290602 SNP was associated with NASH, with an odds ratio (OR) of 2.73 for the T allele. In addition, AST and ALT values of NAFLD patients with the TT genotype were significantly higher than those of patients with the GT or GG allele. However, this association was not further confirmed; a study among the Chinese Han people did not find any association between rs2290602 SNP in *PPARGC1A* gene and NAFLD[133].

Apolipoprotein E and apolipoprotein C-Ⅲ: Apolipoprotein E plays a key role in the metabolism of cholesterol and triglycerides. Indeed, it mediates the binding of the remnant lipoproteins to LDL receptors to favor the clearance of chylomicrons and VLDL from the bloodstream. Two SNPs within the apolipoprotein

E (*APOE*) gene have been identified, resulting in three different alleles (e2, e3, e4) and six *APOE* genotypes with different binding powers[134]. Some association studies investigated the role of *APOE* genotypes on NAFLD/NASH susceptibility with conflicting results. The *APOE* 3/3 genotype was associated with an increased risk of NASH in a cohort of Turkish patients[135], whereas the *APOE* 3/4 genotype had a protective effect[136]. Conversely, Lee *et al*[137] showed no significant difference in *APOE* genotypes distribution among 116 Korean NAFLD patients and 50 controls. However, a protective effect of the e4 allele on fatty liver disease was later shown by Yang *et al*[138] on a large Korean population. Finally, an Italian hospital-based case-control study including 310 NAFLD cases and 422 controls showed that APOE e4 allele carriers had a 2-fold reduction of NAFLD risk compared with e3 homozygotes[139]. The discrepancies between these studies might be attributable to several factors, including different sample sizes, ethnic variability, possible inclusion of alcohol consumers, and lack of clear adjustments for potential metabolic confounders. Apolipoprotein C-Ⅲ is a major constituent of plasma VLDL, chylomicrons, and HDL-C, which inhibits lipoprotein lipase and triglyceride clearance[140]. Two SNPs in the promoter region of the *APOC3* gene - -482C > T and -455T > C, which are in strong linkage disequilibrium with each other - have been repeatedly associated with MS and coronary artery disease[141]. Based on these findings, several studies investigated the association between SNPs of *APOC3* gene and NAFLD occurrence, although with conflicting results. Petersen *et al*[142] firstly reported that ApoC3 T-455C and C-482T promoter SNPs predispose Indian men to liver fat accumulation by altering lipid metabolism and IR. Similar positive results were obtained in Indian[143] and Southern Han Chinese cohorts[144]. However, this association was not further replicated in other studies conducted on Italian[145], British[145], American[146], Finnish[147], German[148], Belgian[149] and Chinese Han[150] subjects. A recent meta-analysis confirmed the absence of a robust association and, therefore, the lack of a causal pathogenetic role of *APOC3* gene polymorphisms in patients with NAFLD[151]. These contrasting findings raise doubts about the methodology and quality of some of these studies, particularly about the methods used to diagnose NAFLD and to adjust for confounders.

Microsomal triglyceride transfer protein: Microsomal transfer tryglicerides protein is a transfer protein involved in apoB-lipoprotein assembly[152]. A large number of common genetic polymorphisms in the microsomal triglyceride transfer protein (*MTTP*) gene have been identified. The G allele of MTTP - 493 G>T polymorphism has been associated with impaired MTTP transcription, and, thus, with a reduced export of triglycerides from hepatocytes and increased susceptibility to NAFLD[153]. Accordingly, the G allele frequency was significantly higher in Japanese patients with NASH, and the severity of NASH was higher in patients with the G/G genotype than in patients with the G/T genotype[154]. Similarly, the -493 G/G genotype was reported to be associated with more severe liver disease and a more atherogenic lipoprotein profile in an Italian cohort[155]. Furthermore, in diabetic French patients, this SNP was associated with elevated ALT as a surrogate marker for NASH[156]. However, other studies did not confirm these reports. Oliveira *et al*[157] did not find any association between - 493 G>T polymorphism and NAFLD in a Brazilian cohort. Similarly, Peng *et al*[158] did not find any significant association between the - 493 G>T polymorphism and the risk for NAFLD in a Chinese Han population, even if other SNPs were found to be associated with NAFLD susceptibility. Specifically, in that study, the rs1800804 T/C was associated with an increased risk of NAFLD, while the rs1057613 A/G and rs3805335 C/T SNPs were associated with a decreased risk. Carulli *et al*[159] found that the distribution of MTTP polymorphisms was not significantly different between NAFLD patients compared with the control group nor associated with clinical or histological characteristics. Finally, a recent meta-analysis including 11 case-control studies with a total of 636 cases and 918 healthy controls revealed that MTP - 493G > T polymorphism was correlated overall with an increased risk of NAFLD among both Caucasian and non-Caucasian populations[160]. However, it should be noted that some of the studies included in the meta-analysis evaluated also featured superimposed NAFLD in HCV-infected patients.

Lipin 1: Lipin 1 is a phosphatase expressed specifically by adipose tissue and liver. It seems to be critically involved in metabolic pathways linking adipose tissue and liver[161]. Several polymorphisms of lipin 1 (LPIN1) have been associated with occurrence of MS and its components[162]. In particular, the LPIN1 rs13412852 T allele was associated with lower body mass index and insulin levels[163]. An Italian study[164] evaluated the LIPIN1 rs13412852 C>T polymorphism in pediatric patients with NAFLD. Investigators demonstrated that the TT genotype, even if underrepresented in pediatric NAFLD patients, was associated with less severe dysplipidemia and a lower prevalence and severity of NASH even after adjustment for genetic - PNPLA3 genotype - and metabolic confounders.

Genetic variants involved in mechanisms of liver injury in NAFLD/NASH

Tumor necrosis factor-α and tumor necrosis factor-related apoptosis-inducing ligand: Tumor necrosis factor-α is an important proinflammatory cytokine involved in the regulation of IR, release of free fatty acids, and induction of apoptosis in hepatocytes under stimuli driven by oxidative stress[165]. Thus, it is not surprising that serum tumor necrosis factor (TNF)-α levels were found to be higher in patients with NASH compared with healthy controls[70] and

that elevated levels have been associated with the occurrence of both NAFLD and NASH[166]. Two polymorphisms of the promoter of *TNF-α* gene have been linked to an increased susceptibility of NAFLD: TNF2 allele (at position -308) and TNFA allele (at position -238)[167,168], both associated with higher TNF-α serum levels[169,170]. However, consistency of this association is still debated[171,172]. Valenti *et al*[167] found that the prevalence of the -238, but not of the -308, TNF-α polymorphism was higher in Italian patients with NAFLD than in controls and that patients with NAFLD positive for both TNF-α polymorphisms had higher IR but a lower number of associated risk factors for steatosis. Furthermore, Tokushige *et al*[171] determined the prevalence of six TNF-α promoter region polymorphisms in a group of Japanese patients with NAFLD and in control subjects. Surprisingly, there were no significant differences in the allele frequencies of any of the six polymorphisms between patients and controls. However, they found that two polymorphisms - -1031C and -863A - were significantly higher in the NASH group compared with subjects with simple steatosis only and that they were associated with an increased homeostasis model assessment for IR (HOMA-IR) score. Finally, negative results were also derived from a prospective cohort of Chinese patients with NAFLD, since *TNF-α* gene polymorphisms were not shown to be associated with NAFLD nor with significant fibrosis[172]. A recent meta-analysis[173] comprising several studies on this topic[167,168,171,172,174-177] concluded that there was a significant difference in TNF-α -238 genotype distribution between NAFLD and control, while there was no clear association between TNF-α -308 genotype and susceptibility for NAFLD. Overall, it is still unclear whether TNF-α polymorphisms are critically involved in NAFLD and/or NASH pathogenesis, probably due to ethnic differences and incomplete control for confounding metabolic factors in most of the studies. Finally, another member of the TNF family, TNF-related apoptosis-inducing ligand (TRAIL), should be mentioned. A Chinese study[178] found that soluble TRAIL levels were significantly higher in NAFLD subjects than in controls and positively correlated with triglyceride concentrations in NAFLD patients and that the AA/TT genotypes of TRAIL at position 1525/1595 conferred a lower risk of NAFLD occurrence and a less severe form of steatosis in NAFLD patients.

Interleukin (IL)-6 and IL-1β: IL-6 is a proinflammatory cytokine produced by adipocytes, hepatocytes, and immune cells, involved in both inflammation and IR[179]. Experimental models have investigated its role in NAFLD pathogenesis and progression, although the results were often contradictory[180-182], whereas certain polymorphisms of the *IL-6* gene were associated with NAFLD susceptibility. A small Italian study[159] found that the *IL-6* -174C variant C - an allele associated with IR, T2D, and MS in some cohorts[183,184] but not in others[185] - was more prevalent in NAFLD than in healthy subjects, associated with increased insulin levels and HOMA-IR, and an independent predictor of NAFLD and NASH. Intriguingly, this finding is in contrast with other studies reporting that it was the *IL-6* -174 G variant that was associated with metabolic abnormalities[186,187]. IL-1 family cytokine members are produced mainly by human adipose tissue; certain IL-1 cytokines - such as IL-1α, IL-1β, IL-18 - have proinflammatory properties, while others - IL-1 receptor antagonist, for example - are anti-inflammatory[188]. Interestingly, IL-1α and IL-1β were shown to have a role in the transition from steatosis to steatohepatitis and liver fibrosis[189]. Based on these findings, Interleukin-1β-511 T/C polymorphism, a functional variant that affects DNA-protein interactions *in vitro*[190], was determined in 63 Japanese NASH patients and 100 healthy volunteers[109]. The authors found that Interleukin-1β -511 T allele frequency and the T/T genotype frequency were significantly higher in NASH patients than in control subjects.

Toll-like receptor 4: Bacterial overgrowth and endotoxemia have recently emerged as two relevant factors in the pathogenesis of NASH[191]. Indeed, the interplay between toll-like receptor 4 (TLR4) and endotoxins results in the release of several mediators capable of favoring hepatic injury and a proinflammatory systemic status[192]. Variants encoded in the ectodomain of the *TLR4* gene, D299G and T399I, have been linked with endotoxin hyporesponsiveness[193] and with possible effects on inflammatory and metabolic disorders like atherosclerosis, IR, MS, and T2D[194,195]. Animal models showed a potential direct link between TLR-4 and Kupffer cells in the pathogenesis of steatohepatitis[192], and, notably, Guo *et al*[196] demonstrated that the D299G and T399I variants were associated with protection from hepatic fibrosis by reducing TLR4-mediated inflammatory and fibrogenic signalling and lowering the apoptotic threshold of activated hepatic stellate cells. Regarding the interaction between NAFLD and TLR-4 polymorphisms in humans, a recent case-control study[197] revealed that the frequency of the heterozygous mutation at position -299 was significantly lower in patients with NAFLD than in controls. However, further studies are needed to clarify the protective role of such polymorphisms in NAFLD pathogenesis and progression.

IL-28B: Several studies repeatedly showed that genetic variations around the *IL-28B* gene strongly predict the spontaneous and treatment-induced clearance of hepatitis C viral infection[198,199]. In particular, IL-28B rs12979860 CC and IL-28B rs8099917 TT genotypes were shown to be closely related to the achievement of a sustained virological response following antiviral therapy[200-202]. Furthermore, other studies revealed a link between IL-28B polymorphisms and the severity

of CHC in terms of steatosis[203,204], necroinflammatory activity[205], and fibrosis[206-208]. Our group reported on a cohort of 160 patients with histological diagnosis of NAFLD that IL-28B rs12979860 CC genotype was associated with the histological severity of liver disease, independently of HOMA and hyperuricemia - well-known risk factors for liver damage in NAFLD[209]. Interestingly, the at-risk CC rs12979860 variant was associated with severe necroinflammation, particularly in subjects with the PNPLA3 G allele, thus leading to hypothesize a potential interplay between these two genes. Such findings were recently confirmed by Eslam *et al*[210] on a large cohort, including 3129 patients with CHC, 555 with chronic hepatitis B, and 488 with NAFLD. The authors demonstrated that rs12979860 genotype acted as a strong predictor of tissue inflammation and fibrosis among all these chronic liver diseases, independent of the underlying etiology. However, Garrett *et al*[211] did not confirm these findings on their North American Caucasian patients with NAFLD, even if they enrolled a cohort of severe obese NAFLD patients evaluated for bariatric surgery, and, therefore, very different from our cohort. Overall, these data suggest an effect of *IL-28B CC* genotype in patients at lower metabolic risk only, and not in obese patients, where the burden of metabolic alterations on NAFLD severity likely overcomes the role of the genetic background.

Superoxide dismutase 2 and cytochrome P450 2E1: The superoxide dismutase 2 (*SOD2*) gene encodes for the mitochondrial enzyme manganese-dependent superoxide dismutase, a protein that protects cells from injury induced by superoxide radicals[212]. Interestingly, oxidative stress is regarded as a relevant factor involved into the transition from simple steatosis to steatohepatitis[213]. A common polymorphism in the *SOD2* gene - C47T, rs4880 - has been related to relatively efficient protein function by *in vitro* studies[214,215], and *SOD2* variants have been investigated in settings of alcoholic liver disease with inconsistent results[216,217]. Regarding the role of SOD2 C47T polymorphism in NAFLD, a small study performed on 63 Japanese subjects revealed an increased prevalence of the lower activity homozygous T genotype among patients with NASH compared with controls[154]. Similar conclusions were drawn from a cohort of obese Egyptian children with steatosis or NASH[218]. Al-Serri *et al*[219] performed a two-step analysis of the relevance of this SNP in NAFLD: the preferential transmission of alleles from parents to affected children in 71 family trios and a classical case-control study involving a cohort of 502 European patients with fatty liver. Investigators demonstrated that *SOD2* genotype - together with *PNPLA3* genotype, T2D, and histological severity of NASH - was associated with an advanced stage of fibrosis. Conversely, a Chinese study did not find any significant difference in the frequencies of the three SOD2 genotypes among patients and controls but highlighted how the frequency of the SOD2 C variant was higher in the NASH group than in subjects with simple steatosis and in controls[220]. The same study evaluated another gene potentially involved in NAFLD pathogenesis: cytochrome P450 2E1 (CYP2E1), encoding for cytochrome P450 2E1 - another enzyme related to superoxide radicals in humans. Indeed, induction of CYP2E1 is a central process involved in generating oxidative stress in both alcoholic and nonalcoholic steatohepatitis[221]. However, evidence about a potential role of *CYP2E1* gene SNPs in NAFLD pathogenesis are elusive. On the one hand, the above mentioned study[220] did not report any association between the CYP2E1 -1053C>T variation (*1/*5 - rs2031920) and increased susceptibility to NAFLD or NASH in Chinese subjects; on the other hand, Varela *et al*[222] found that the CYP2E1 *5 variant was positively associated with liver injury in obese women with NASH, and similar positive results were also found on a Chinese population[223]. It is likely that ethnic differences and the incomplete understanding of the real effect of SOD2 and CYP2E1 genotypes on related enzymatic activities could be the main reasons underlying these conflicting results.

Uncoupling protein 3 and uncoupling protein 2: Uncoupling protein 3 is a mitochondrial anion carrier selectively expressed in skeletal muscle - the major site of thermogenesis in humans - involved in the metabolism of superoxide radicals and in the modulation of energy and lipid homeostasis[224-226]. The rs1800849 -55C/T polymorphism of uncoupling protein (UCP) 3 has been associated with an increased susceptibility to T2D and obesity and with an atherogenic lipid profile[227-229]. Interestingly, the rs1800849 UCP3 -55CT genotype was also associated with IR, increased adiponectin levels, the presence of moderate-severe steatosis, and NASH in a small Spanish study[230]. Furthermore, an interesting Chinese paper aiming to evaluate the frequency of four nonsynonymous SNPs in the *UCP3* gene in a pediatric cohort found a higher prevalence of another variant - rs11235972 GG genotype - among patients with NAFLD compared with control subjects[231]. Similar to UCP3, UCP2 is involved in the regulation of mitochondrial lipid efflux and oxidative metabolism. Its increased hepatic expression has been reported both in experimental models and in NASH patients as a protective mechanism against liver injury progression[232]. A promoter region polymorphism of UCP2 - -866 G>A variant - is able to influence the extrahepatic expression of UCP2 and insulin release and sensitivity, although the overall metabolic impact is still controversial[233]. A recent Italian paper investigated the role of this SNP in patients who underwent liver biopsy for suspected NASH[234]. UCP2 -866 A/A genotype was associated with a reduced risk of NASH after adjustment for age, sex, body

mass index, impaired fasting glucose or diabetes, and PNPLA3 I148M allele and with a reduced risk of steatosis grade G2-G3 and NASH in patients without, but not in those with, impaired fasting glucose/diabetes. Concerning the metabolic traits, the UCP2 A/A genotype was associated with higher total serum cholesterol levels but not with serum HDL, triglycerides or impaired fasting glucose/diabetes. Overall, SNPs in *UCP* genes may confer susceptibility or protection to NAFLD/NASH, even if further evidence needs to be provided.

Methylenetetrahydrofolate reductase: Homocysteine is an intermediate amino acid formed during methionine metabolism in the liver. Today, hyperhomocysteinemia is regarded as a risk factor for liver diseases *via* the promotion of oxidative and endoplasmic reticulum stress, and the activation of proinflammatory factors[235,236]. Methylenetetrahydrofolate reductase (MTHFR) catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a metabolic pathway fundamental for the methylation of homocysteine to methionine. Several genetic polymorphisms in the *MTHFR* gene have been identified, and among them, the C677T polymorphism (rs1801133) and the A1298C (rs1801131) - inducing both a reduction of MTHFR activity - were extensively investigated[237,238] in the setting of NAFLD[239-242]. Sazci *et al*[239] analyzed the frequency of C677T and A1298C polymorphisms of *MTHFR* gene in a Turkish cohort comprising 57 NASH patients and 324 healthy controls, showing that the MTHFR 1298C allele in all NASH patients, the C677C/C1298C compound genotype in women, and the C677C/A1298C compound genotype in men were genetic risk factors for NASH. Similarly, Catalano *et al*[240] recently identified the MTHFR A1298C heterozygous polymorphisms as a weak predictor for NAFLD severity in an Italian cohort. However, the relationship between MTHFR polymorphisms and NAFLD remains controversial. Franco Brochado *et al*[241] did not find any association between the MTHFR C677T and A1298C polymorphisms and NAFLD and its severity. Similarly, Serin *et al*[242] showed that the MTHFR C677T polymorphism was not a risk factor for NAFLD in their Turkish cohort. As a consequence, more rigorous work needs to be performed in this field.

Glutamate-cysteine ligase catalytic subunit: The glutamate-cysteine ligase catalytic subunit (*GCLC*) gene codes the catalytic subunit of the heterodimeric γ-Glutamate-cysteine ligase, the limiting enzyme in the formation of glutathione, a relevant endogen antioxidant. The base T in the position -129, as opposed to base C, determines a sharp decrease in the promoter activity of the *GCLC* gene and was identified as a significant independent risk factor for myocardial infarction in a Japanese population[243]. In addition, mitochondrial glutathione depletion has been associated with the development of alcoholic steatohepatitis due to the increased sensitivity of hepatocytes to the pro-oxidant effects of cytokines generated by ethanol metabolism[244]. Interestingly, Oliveira *et al*[157] found that, among 131 biopsy-proven NAFLD patients, the presence of at least one T allele in the -129 C/T polymorphism of the *GCLC* gene was independently associated with NASH detection, with an OR of 12.14. Thus, such polymorphism could be an important factor in the development of liver injury mediated by oxidative stress.

Hemochromatosis and trans-membrane protease serine 6: Human hemochromatosis protein (HFE) is crucial for the regulation of iron homeostasis *via* modulation of the expression of hepcidin[245]. Excessive hepatic iron deposition is a frequent histological feature of NASH, and it has been investigated as a potential contributor to oxidative stress in the liver, and thus as a second hit promoter[246]. In this regard, even if the C282Y and H63D mutations of the *HFE* gene - common in Caucasians and responsible for most cases of hereditary hemochromatosis - are well-known causes of potential iron overload, their prevalence and relevance in patients with NAFLD have been variable, depending on the examined cohorts. The first reports about the association between HFE mutations and NAFLD came in the late 1990s and showed a positive correlation between these two conditions[247,248]. Later, Lee *et al*[249] identified the presence of H63D mutation as an independent factor associated with NAFLD in the Korean population, and Nelson *et al*[250] suggested that the presence of the C282Y mutation was a risk factor for the development of advanced hepatic fibrosis among American Caucasian patients with NASH. Nonetheless, other studies have not confirmed such associations. Indeed, even if several reports suggested that increased ferritin levels may be markers of histological damage, the HFE mutations did not consistently contribute to hepatic fibrosis in NAFLD[251] nor to its susceptibility[252]. The poor relevance of HFE mutations in NAFLD have been resumed by a recent meta-analysis including 610 cases and 7298 controls[253]: authors found no associations between iron-overloading HFE mutations and NAFLD susceptibility or severity. However, other genetic variants influencing iron deposition may be involved in NAFLD/NASH pathogenesis. Beta-globin mutations have been identified as a good genetic predictor of parenchymal iron overload in Italian patients with NAFLD and have been associated with a two-fold higher risk of severe fibrosis[254]. More recently, the rs855791 C>T polymorphism of the trans-membrane protease serine 6 (*TMPRSS6*) gene - encoding for matriptase-2, which cleaves the membrane-bound hemojuvelin, a co-receptor required for hepcidin expression in the liver[255] - has been associated with

lower hepatic iron stores, ferritin levels, and ballooning in 216 patients with histological NAFLD[256].

Kruppel-like factor 6: The kruppel-like factor 6 (KLFs) are a family of zinc finger-containing transcriptional factors that regulate cellular processes, such as proliferation, differentiation, and apoptosis[257]. In the liver, injury and/or cytokines are able to induce *KLF6* gene expression, which in turn plays an essential role in the transactivation of several genes involved in the development of liver fibrosis, mainly *via* the activation of hepatic stellate cells[258]. Miele *et al*[259] reported the association between a functional polymorphism in the *KLF6* gene - IVS1-27G>A SNP (rs3750861) - and the severity of NAFLD. In particular, they demonstrated increased levels of total and wild type KLF6 expression in patients with NAFLD and higher steatosis, inflammation, and fibrosis, whereas KLF6 IVS1-27G>A SNP was associated with reduced fibrosis, and thus, acted as a protective factor against NASH progression. Intriguingly, the effects of KLF6 genotype on NAFLD/NASH pathogenesis may also involve the modulation of metabolic pathways: Bechmann *et al*[260] observed that KLF6 IVS1-27G wild-type allele was associated with increased fasting glucose and insulin levels and with decreased hepatic insulin sensitivity *via* the reduced expression of glucokinase. KLF6 increased PPARα activity, whereas KLF6 loss led to PPARα repression and attenuation of lipid and glucose abnormalities[261].

Transforming growth factor-β1, angiotensin Ⅱ, and angiotensin Ⅱ type 1 receptor: The transforming growth factor (TGF)-β1 is a well-known promoter of hepatic fibrosis that contributes to the activation of hepatic stellate cells[262]. TGF-β1 production can be stimulated by angiotensin Ⅱ (AT Ⅱ), part of the renin-angiotensin system that has been advocated as a potential inducer of extracellular matrix accumulation[263]. A higher frequency of a pro-fibrotic TGF-β1 SNP (Arg/Arg at codon 25) has been identified in patients with hypertension compared with controls[264]. Furthermore, this TGF-β1 SNP and an ATⅡ variant in the promoter region of the gene (AT-6 G>A), leading to a higher transcription of AT, were both associated with increased hepatic fibrosis in patients with CHC[265]. Based on these findings, Dixon *et al*[266] investigated these two polymorphisms in a group of severely obese patients with NASH. The investigators found a positive association between AT-6 A/A polymorphism and severe fibrosis, even if such correlation was lost after correction for gender. However, patients with both high ATⅡ and TGF-β1 producing polymorphisms had a higher risk of advanced fibrosis. In addition, animal models had demonstrated that the Angiotensin Ⅱ Type 1 Receptor (*AGTR1*) gene could be implicated in the susceptibility to NAFLD[267]. In this line, none of the five variants of the *AGTR1* gene were associated with susceptibility to NAFLD in a multi-ethnic Asiatic cohort composed of Malayan, Indian, and Chinese subjects, with the exception of the Indian subgroup, where the rs2276736, rs3772630, and rs3772627 were found to be protective against NAFLD and NASH[268]. Furthermore, five SNPs of *AGTR1* gene (rs3772622, rs3772633, rs2276736, rs3772630, and rs3772627) were significantly associated with NAFLD in a Japanese cohort[269]. All in all, the potential involvement of the renin-angiotensin system in NAFLD/NASH pathogenesis is still unclear, and further research is needed.

CONCLUSION

In the complex pathogenetic puzzle of NAFLD, genes clearly act as major disease modifiers affecting NAFLD occurrence and severity and sometimes cardiovascular risk as well. To date, the PNPLA3 gene variant is the most validated susceptibility factor for steatosis, NASH, fibrosis, and HCC, despite a number of other genetic variants contributing to liver damage. However, even if the identification of these variants helped us to understand better NAFLD in terms of both clinical phenotypes and pathogenetic mechanisms, their utility in clinical practice and in the individual patients is far from being relevant. Therefore, further efforts should be done to generate a genetic map useful to stratify the hepatic and non-hepatic risk of NAFLD patients and to define better therapeutic approaches in terms of both lifestyle intervention and new pharmacological therapies.

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MINIREVIEWS

Active vaccination to prevent *de novo* hepatitis B virus infection in liver transplantation

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Abstract

The shortage of organ donors mandates the use of liver allograft from anti-HBc(+) donors, especially in areas highly endemic for hepatitis B virus (HBV) infection. The incidence of *de novo* hepatitis B infection (DNH) is over 30%-70% among recipients of hepatitis B core antibody (HBcAb) (+) grafts without any prophylaxis after liver transplantation (LT). Systematic reviews showed that prophylactic therapy [lamivudine and/or hepatitits B immunoglobulin (HBIG)] dramatically reduces the probability of DNH. However, there are limited studies regarding the effects of active immunization to prevent DNH, and the role of active vaccination is not well-defined. This review focuses on the feasibility and efficacy of pre- and post-LT HBV vaccination to prevent DNH in HBsAg(-) recipient using HBcAb(+) grafts. The presence of HBsAb in combination with lamivudine or HBIG results in lower incidence of DNH and may reduce the requirement of HBIG. There was a trend towards decreasing incidence of DNH with higher titers of HBsAb. High titers of HBsAb (> 1000 IU/L) achieved after repeated vaccination could eliminate the necessity for additional antiviral prophylaxis in pediatric recipients. In summary, active vaccination with adequate HBsAb titer is a feasible, cost-effective strategy to prevent DNH in recipients of HBcAb(+) grafts. HBV vaccination is advised for candidates on waiting list and for recipients after withdrawal of steroids and onset of low dose immunosuppression after transplantation.

Key words: *De novo* hepatitis B; Vaccination; Liver transplantation; Core antibody positive donor

Core tip: *De novo* hepatitis B virus infection (DNH) can both result in significant morbidity and reduced graft survival after liver transplantation. Utilization of hepatitis B core antibody(+) grafts may increase the risk of DNH. Different approaches to mitigate this risk have been described. There is no widespread consensus regarding the prophylactic measures to reduce the incidence of DNH by active immunization.

This review examines the important published studies on DNH, and presents the clinically relevant points in a lucid manner. It also presents an algorithm which is simple to follow, and which has been validated in pediatric patients at our center.

Lin CC, Yong CC, Chen CL. Active vaccination to prevent *de novo* hepatitis B virus infection in liver transplantation. *World J Gastroenterol* 2015; 21(39): 11112-11117 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i39/11112.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i39.11112

INTRODUCTION

There is a wide disparity between the number of patients awaiting liver transplantation (LT) and the pool of available donors. Strategies such as living donor LT and using extended criteria donors have been utilized to increase the number of liver grafts. Additionally, many LT programs use liver grafts from hepatitis B core antibody (HBcAb)-positive donors to increase donor organ availability. The acquisition of hepatitis B virus (HBV) infection after transplantation in recipients who are hepatitis B surface antigen (HBsAg)-negative before transplantation has been recognized[1]. The incidence of *de novo* HBV infection (DNH) among patients receiving HBcAb(-) grafts is low (0%-1.7%) but unacceptably high (38%-100%) among recipients receiving HBcAb(+) grafts without prophylaxis[2-5]. The mechanism has been well-established that HBV DNA may persist in the serum and liver in low replicating or non-replicative forms following serologic recovery from HBV infection, thereby presenting a risk of DNH[6]. Hence, some centers have suggested to exclude these grafts from HBcAb(+) donors or to limit its use in selected recipients[7] . This strategy is not practical in endemic areas for HBV infection[8,9].

Several strategies have been recommended to prevent DNH in non-HBV recipients who receive HBcAb(+) grafts. Hepatitis B immunoglobin (HBIG) and/or lamivudine have been most commonly used for prophylaxis. Recently, the results have been extensively reviewed[10,11]. However, the results of active immunization are still not well-documented due to heterogenous data resources and limited case numbers. In this review, we mostly focus on the role of active vaccination before and after LT to prevent DNH.

POST-TRANSPLANT PROPHYLAXIS AGAINST *DE NOVO* HBV INFECTION

Lamivudine monoprophylaxis

Studies show that lamivudine monoprophylaxis (100-150 mg/d for long periods) has been an effective strategy against DNH. During a median follow-up of 25 mo (range: 1-69 mo), the incidence of DNH was observed in 2.6% of recipients: 4.0% in recipients with past HBV infection, 3.4% in HBV naive recipients and 0% in recipients with successful pre-LT vaccination[10]. New generations of nucleos(t)ide analogs have been used to replace lamivudine in order to further reduce the probability of DNH. Tenofovir and entecavir seem to be more potent than lamivudine and adefovir in limited cases[12]. It is worth noting that the lowest probability was observed among recipients with the presence of HBsAb despite no statistical difference. HBV naive recipients were more likely to develop DNH after nucleos(t)ide discontinuation. These observations suggest that the presence of HBsAb alone may not completely prevent DNH but plays a role in combination with antiviral agents to prevent DNH.

HBIG monoprophylaxis

During a median follow-up of 31 mo (range: 3-86 mo), 18% of recipients with HBIG monoprophylaxis developed DNH, of which 27% had discontinued HBIG and 11% had low serum anti-HBs levels (< 50 IU/mL) despite HBIG administration[10]. As to HBV status, DNH developed in 27% of HBV naive recipients and 5.8% of recipients with past HBV infection (P = 0.10) during a median follow-up of 30 mo and 19 mo, respectively. Of importance, DNH developed in none of five recipients with successful pre-LT vaccination during a median follow-up of 35 mo. Although the impact of the recipients' HBsAb status could not be determined, there was a trend toward decreasing incidence of DNH in combination with the presence of HBsAb. Moreover, a smaller number of recipients with cessation of HBIG had much higher incidence of DNH than recipients with long term HBIG administration (P = 0.0002)[11]. Most centers use HBIG indefinitely to prevent DNH; post-LT vaccination has therefore been utilized to replace the use of HBIG[13].

HBIG and lamivudine combination therapy

Since the combination of HBIG with lamivudine was the most widely used treatment approach to prevent post-LT HBV recurrence in patients transplanted for HBV-related liver disease, it was also used as prophylaxis against DNH. However, given the low probability of DNH with lamivudine or HBIG alone, the benefit of HBIG with lamivudine combined prophylaxis was similar to lamivudine alone[10]. There is no clear evidence showing the necessity of using combination therapy.

Pre-LT recipient HBV status

HBV naive recipients receiving HBcAb(+) grafts are highly susceptible to develop DNH without prophylaxis. Recipients of HBcAb(+) grafts had DNH rates of 18% without prophylaxis and 0% with prophylaxis. Recipients with HBsAb and received HBcAb(+) grafts had DNH rates of 4% without prophylaxis and 3% with

prophylaxis; whereas, recipients with HBcAb positivity alone had DNH rates of 14% without prophylaxis and 3% with prophylaxis (P = 0.21)[11]. In addition, some centers allocated HBcAb(+) grafts to HBsAb(+) candidates if possible to minimize DNH[14]. These data suggest that the presence of HBsAb either from past infection or active vaccination provides some additional benefits against DNH.

Active immunization against DNH

The long-term use of lamivudine may raise the concern of mutant strain and HBIG is a costly and inconvenient regimen. Non-compliance is a significant factor for failure of prophylaxis. The difference between innate and exogenously administered antibody is unknown. The acquisition of immunity through active vaccination is preferred if both are equally effective. Active vaccination has been suggested against DNH. Although HBsAb alone is not able to eliminate DNH completely, its presence reduces the probability of DNH either in lamivudine or HBIG regimen. In addition, a study indicated that the level of pre-LT HBsAb in children was associated with the response of post-LT booster vaccine[15]. Therefore, pre-transplant active vaccination for candidates in waiting list is fundamental to avoid DNH.

Pre-transplant vaccination

HBV vaccines are extremely safe and have an efficacy of more than 90% in the general population; the response rate is lower in cirrhotic patients due to the impairment in T-cell dependent function. The results of vaccination in pre-LT candidates have been very disappointing, with response rates of 20%-30%[16]. The accelerated protocol, double-dose schedule and other more immunologic formulation have been proposed to increase the response. A study reported that vaccination with this double-dose schedule can achieve 65% response rate in candidates before transplantation[17]. Age plays an important role in the response to HBV vaccine; especially many pediatric patients for LT have non-parenchymal, cholestatic diseases. In our pediatric series, 72% of recipients were positive for HBsAb at the initial evaluation for transplantation due to the national vaccination program. After pre-LT booster vaccination, all recipients were positive for HBsAb with a median of 784 (8-18736) (IU/L)[18]. Likewise, 63% of our adult non-HBV patients were positive for HBsAb at the initial evaluation and 93% were positive after double-dose booster vaccination before transplant (unpublished data).

Post-LT vaccination

Although the presence of HBsAb in recipients is protective to a certain degree, a small number of recipients with pre-LT serologic immunity do develop DNH, particularly those whose immunity was based on vaccination (HBsAb alone) rather than prior exposure to HBV[11]. Active vaccination was not absolutely effective to prevent DNH, because it was impossible to maintain an optimal HBsAb titer to prevent DNH during the early post-LT period.

The HBV vaccination response rates are lower during the early post-LT period with intense immunosuppression. Although a double-dose schedule was used, it was difficult to maintain an adequate HBsAb titer[19]. It is generally accepted that the immune system is less capable of mounting effective immune responses in recipients receiving high dose immunosuppression. T- and B-cell responses to antigenic stimulation are impaired through blockade of cellular proliferation by calcineurin inhibitors and steroids as well as by inhibition of cytokine production[20]. To optimize the response, the timing of vaccination appears to be critical. The first 6 mo post-LT appears to be a period where mounting immune responses are lowest since the recipients are usually on high dose immunosuppression.

The key factors for successful vaccination are the vaccine doses, the rapid schedule and administration during the low immunosuppression period[21]. As a general rule, vaccination should be administered when the dosages of immunosuppressants have been reduced post-LT, especially after steroid withdrawal. Repeated vaccination after LT in pediatric patients at our center resulted in HBsAb titers higher than 100 IU/L in about 80% cases and over 1000 IU/L in 40% cases[18]. Similarly, all adult recipients were positive for HBsAb, and approximately 80% patients achieved titers higher than 100 IU/L and 38% had titers over 1000 IU/L (unpublished data). It is not uncommon to observe decline of protective antibodies after LT, particularly if HBsAb is acquired by vaccination. Therefore, routine HBV booster vaccination is advised after steroid withdrawal and when recipients are on low-dose immunosuppression after transplant.

How high of HBsAb is enough

HBsAb level > 10 IU/L after vaccination is considered protective in immunocompetent patients. A plausible explanation for recipients developing DNH despite presence of HBsAb may be that their titers were insufficient to confer protection. HBIG prophylaxis to maintain HBsAb level > 500 IU/L against DNH has been advised[22,23]. However, the adequate titer of HBsAb to be achieved by vaccination, in order to protect immunosuppressed recipients against DNH is still not well defined (Table 1). HBsAb < 20 IU/L was obviously not protective against DNH[19,24]. Su *et al*[25] found that HBsAb > 200 IU/L by vaccination significantly protects pediatric recipients receiving HBcAb(+) positive grafts from DNH, reducing its incidence to 11%. Another group demonstrated the similar results by HBIG administration[26]. In our study, in combination with lamivudine, vaccination with HBsAb > 1000 IU/L significantly reduced the incidence

Table 1 Published studies with HBsAb titers in patients receiving HBcAb(+) grafts

Study	Patients	Lamivudine/HBIG	Vaccination Pre/post-LT	HBsAb cutoff (IU/L)	DNH	Follow-up (mo)
Chang *et al*[24]	9/pediatric	HBIG	Yes/Yes	20	50% *vs* 0%[1]	26
Lin *et al*[18]	30/pediatric	lamivudine	Yes/Yes	1000	15.7% *vs* 0%	57
Soejima *et al*[13]	11/adult	HBIG	No/Yes	1000	0%[2]	15
Park *et al*[26]	14/pediatric	HBIG	No/Yes	100	7.1%[3]	26.5
Su *et al*[25]	36/pediatric	N	Yes/Yes	200	30.7% *vs* 4.3%	52
Liu *et al*[28]	41/adult	Lamivudine	Yes/Yes	1000	6.4% *vs* 0%	52
Lin *et al*[27]	34/pediatric	N	Yes/Yes	1000	0%[4]	65

[1]Six out of 7 patients had > 200 IU/L; [2]All > 1000 IU/L, 4 patients with cessation of HBIG; [3]All > 100 IU/L, one patient with 362 IU/L of HBsAb and escape mutant; [4]All > 1000 IU/L without additional prophylaxis. HBIG: Hepatitis B immunoglobulin; DNH: *de novo* hepatitis B infection; LT: Liver transplantation.

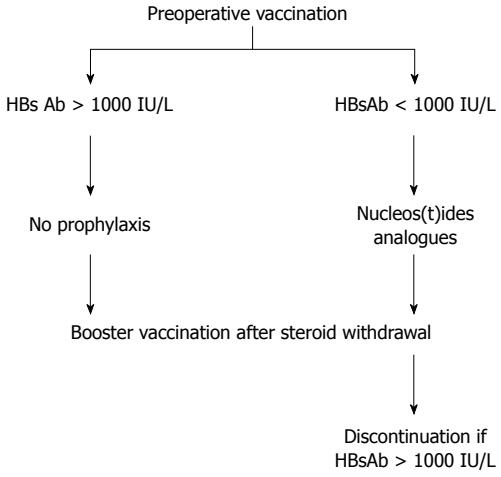

Figure 1 Algorithm for prophylaxis to prevent *de novo* hepatitis B virus for recipients of HBcAb(+) grafts. For recipients with pretransplant HBsAb > 1000 IU/L, no antiviral prophylaxis is required. Recipients require nucleos(t)ide analogs if HBsAb is < 1000 IU/L. Post-transplant vaccination is given after steroid withdrawal. Nucleos(t)ide analogs may be discontinued if HBsAb is > 1000 IU/L.

of DNH from 15.4% to 0% in pediatric patients[18]. Notably, without antiviral agent prophylaxis, HBsAb > 1000 IU/L completely prevented the development of DNH in pediatric patients with a follow-up of more than 5 years[27]. Likewise, there was no case of DNH in 10 (24.3%) adult recipients with HBsAb > 1000 IU/L[28]. It is generally accepted that the level of HBsAb naturally declines and may even drop rapidly during heavy immunosuppression. These observations theoretically suggest that the higher the levels of HBsAb that can be achieved, the lower the incidence of DNH. In over 107 recipients with HBsAb > 1000 IU/L (Table 1), only one (approximately 0.9%) developed DNH due to escape mutant when post-LT HBsAb dropped to 362 IU/L[26].

Escape mutant

Another major concern for vaccination is the escape mutant of "*a*" determinant within HBsAg which can develop after HBV vaccination and use of HBIG post-LT[29,30]. A change of amino acids in the "a" determinant could lead to a structural variation in the epitope of the surface antigen recognized by HBsAb, which affects virus binding with a loss of immunoreactivity despite the presence of HBsAb. The incidence of escape mutant was below 4% in HBV-infected infants who received HBV vaccine or HBIG to an HBV-infected mother but up to 11%-66% in LT recipients who experienced HBV reinfection after HBIG prophylaxis[31]. Although the incidence of escape mutant in transplant recipients is uncertain, it would be lower compared to the administration of HBIG. Thus, we believe that the benefits of HBV vaccination for prophylaxis against DNH outweigh its potential side effects.

Donor HBsAb status

Whether it is possible to obtain adaptive transfer from donor HBsAb to recipient is not well-established. Both animal and human studies have demonstrated the adoptive transfer of immunity against HBV through LT that may be attributed to the presence of HBV-specific immunocompetent cells of donor origin in liver grafts[32]. However, most studies showed that donor HBsAb did not provide any additional benefits against DNH[18,25].

CONCLUSION

Despite the scarcity of evidence-based data due to heterogeneous prophylactic strategy and limited case numbers, some conclusions can be drawn based on these reviews. The presence of HBsAb in combination with antiviral agents or HBIG reduces the incidence of DNH. High titers of HBsAb (> 1000 IU/L) is an effective strategy to prevent DNH without additional prophylaxis, which can be achieved by repeated pre- and post-LT booster vaccination. Therefore, we recommend the following prophylactic strategy to prevent DNH in LT recipients receiving HBcAb(+) grafts (Figure 1). For recipients with pre-LT HBsAb titers > 1000 IU/L, no antiviral agent prophylaxis is required but only a booster vaccination after steroid withdrawal. However, recipients with HBsAb titers below 1000 IU/L require prophylaxis with nucleos(t)ide analogs in addition to booster HBV vaccinations after steroid withdrawal. Nucleos(t)ide analogs may be discontinued when the HBsAb is > 1000 IU/L after post-LT booster vaccination.

In conclusion, active vaccination in combination with nucelos(t)ide analogues is a simple and cost-effective strategy to prevent DNH. This strategy may reduce the incidence of DNH even if high titers of HBsAb are not achieved. It is advised that active vaccination be administered for non-HBV patients before and after transplantation.

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ORIGINAL ARTICLE

Basic Study

Hepatic differentiation of rat induced pluripotent stem cells *in vitro*

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Author contributions: Sun C performed the experiments, analyzed the data and drafted the article; Hu JJ performed the experiments; Pan Q and Cao Y analyzed the data; Fan JG supervised the study and revised the article; Li GM conceived and designed the study.

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Abstract

AIM: To show the efficient generation of hepatocyte-like cells (HLCs) differentiated from the induced pluripotent stem cells (iPSCs) of rats.

METHODS: Hepatic differentiation was achieved using a three-step protocol with several growth factors. First, rat iPSCs were differentiated into definitive endoderm cells using Activin A and Wnt3a treatment. Then fibroblast growth factor 4 and bone morphogenetic protein 2 were added to the culture medium and used to induce hepatic differentiation. Finally, hepatocyte growth factor, Oncostatin M and dexamethasone were used for hepatic maturation. The liver-related markers and functions of HLCs were assessed at the gene and protein levels.

RESULTS: After endodermal induction, the differentiated cells expressed endodermal markers forkhead box protein A2 and SRY-box containing gene 17 at the mRNA and protein levels. After 20 d of culture, the iPSCs were differentiated into HLCs. These differentiated cells expressed hepatic markers including α-fetoprotein, albumin CK8, CK18, CK19, and transcription factor HNF-4α. In addition, the cells expressed functional proteins such as α1-antitrypsin, cytochrome P450 1A2 and CYP 3A4. They acted like healthy hepatic cells, storing glycogen and taking up indocyanine green and low-density lipoproteins. Also, the rates of urea synthesis (20 d 1.202 ± 0.080 mg/dL *vs* 0 d 0.317 ± 0.021 mg/dL, $P < 0.01$) and albumin

secretion (20 d 1.601 ± 0.102 mg/dL *vs* 0 d 0.313 ± 0.015 mg/dL, $P < 0.01$) increased significantly as differentiation progressed.

CONCLUSION: Rat iPSCs can differentiate into HLCs rapidly and efficiently. These differentiated cells may be an attractive resource for treatment of end-stage liver disease.

Key words: Hepatic differentiation; Induced pluripotent stem cells; Hepatocyte-like cells; Rat

Core tip: Induced pluripotent stem cells (iPSCs) can differentiate into many kinds of cells, including hepatocytes. However, the most important animal model for studying human diseases, especially liver diseases, is the rat model. This study is the first to show the efficient generation of hepatocyte-like cells (HLCs) differentiated from the iPSCs of rats. Hepatic differentiation was achieved using a three-step protocol. Rat iPSCs were differentiated into HLCs after 20 d of culture. These differentiated cells expressed hepatic markers and exhibited functional hepatic characteristics. These differentiated cells may be an attractive resource for treatment of liver disease.

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INTRODUCTION

So far, there has been no ideal treatment for end-stage liver disease attributable to chronic hepatitis or cirrhosis. Recent studies have suggested that differentiated hepatocytes derived from transplanted stem cells may be a suitable form of therapy[1,2]. Embryonic stem cells (ESCs) and fetal liver epithelial cells can form mature hepatocytes *in vivo*. This might reduce the severity of hepatic fibrosis[3,4]. However, the collection of these cells from human embryos involves certain ethical and political issues, which limit their use in clinical settings. According to a previous study, bone marrow-derived liver stem cells may suppress hepatic fibrosis and ameliorate liver function[5]. However, their ability to proliferate is much poorer than that of ESCs, leaving them unable to produce a large number of cells[6]. Bone marrow contains several different components. This low purity produces a non-homogeneous crop of differentiated cells. This affects the treatment after transplantation[7].

Induced pluripotent stem cells (iPSCs) sidestep most of the ethical issues surrounding ESCs. They can produce functional hepatocytes and provide new seed cells for the treatment of hepatic diseases[8,9]. Studies have shown that iPSCs can differentiate into neural cells[10,11], osteogenic cells[12,13], cardiac cells[14,15], vascular cells[16,17] and pancreatic cells[18,19]. The differentiation of iPSCs into hepatic lineages has been observed in humans and mice using similar protocols[20-22]. However, the laboratory rat was the first mammalian species domesticated for scientific research, and the rat model is commonly used in research involving hepatic diseases. So far, no reports have been published on the differentiation of rat iPSCs into hepatocyte-like cells (HLCs). In order to develop such technologies in rat models, a method suitable for the differentiation of rat iPSCs into HLCs is here presented and the necessary conditions are given.

In the present study, rat iPSCs were differentiated into HLCs using several growth factors. The characteristics and functions of the HLCs were evaluated. These cells might be suitable for use as a cell resource for transplantation into model animals with liver diseases.

MATERIALS AND METHODS

Culture of rat iPSCs

Rat iPSC line M13 was provided by the research group led by Professor Lei Xiao of the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences[23]. Rat iPSCs were cultured with a feeder layer of irradiated mouse embryonic fibroblasts (MEF) in iPSC medium (DMEM/F12 containing 20% knockout serum replacement, 2 mmol/L L-glutamine, 1% nonessential amino acids, 0.1 mmol/L β-mercaptoethanol (all from Invitrogen/Gibco, Grand Island, NY, United States) in a humid atmosphere containing 5% CO_2. Before differentiation, the cells were cultured on gelatin-coated tissue culture dishes with MEF-conditioned medium.

Hepatic differentiation of rat iPSCs

During the first 5 d, rat iPSCs were cultured in the Roswell Park Memorial Institute 1640 medium (Invitrogen/Gibco) with 25 ng/mL Wnt3a and 100 ng/mL Activin A (both from R and D Systems, Minneapolis, MN, United States) for endodermal induction. Next, the inductive factors were replaced with 30 ng/mL fibroblast growth factor 4 (FGF4, R and D Systems) and 20 ng/mL bone morphogenetic protein 2 (BMP2, R and D Systems) for 5 d. Finally, during the maturation step, the differentiated cells were incubated in the same basal medium containing 10 ng/mL Oncostatin M (OSM, R and D Systems), 20 ng/mL hepatocyte growth factor (HGF, R and D Systems), and 0.1 μmol/L dexamethasone (Dex, Sigma-Aldrich, St. Louis, MO, United States) for another 10 d.

Immunofluorescence staining

On day 5 after endodermal induction, differentiated cells were tested for SRY-box containing gene 17 (SOX17)

Table 1 Primers, GenBank accession numbers, and amplicon size of genes for reverse transcription-polymerase chain reaction

Gene	Primer	Accession Number	Amplicon size (bp)
FOXA2	TGTCAGGAGCACAAGCGAGG (F) GGGTGGTTGAAGGCGTAATGGTG (R)	NM-012743.1	194 bp
SOX17	GGCACGGAACCCAACCAGC (F) CAGTCGTGTCCCTGGTAGGGAAGAC (R)	NM-001107902.1	119 bp
AFP	TCTGAAACGCCATCGAAATGCC (F) AATGTAAATGTCGGCCAGTCCCT (R)	NM-012493.2	285 bp
ALB	GGCACAGTGCTTGCAGAATTTCAG (F) CACAGACGGTTCAGGATGGCAG (R)	NM-134326.2	272 bp
CK8	TGAAGGATGCCAATGCCAAGCTG (F) AACTCAGTCCTCCTGCGTAGCC (R)	NM-199390.1	237 bp
CK18	ACGATTTCAGTCTCAACGACGCC (F) TCCCTTCTTCTGAGCCTTAGTGCC (R)	NM-053976.1	147 bp
CK19	GCTCAGCATGAAAGCTGCCCT (F) CGCACTGGTAGCAAGGTAGGAG (R)	NM-199498.1	299 bp
HNF4α	GATGTGCTGCTCCTAGGCAATGAC (F) CTGCCGGTCGTTGATGTAATCCTC (R)	NM-001270931.1	261 bp
GADPH	CCTTCATTGACCTCAACTAC (F) GGAAGGCCATGCCAGTGAGC (R)	NM-017008.4	594 bp

and forkhead box protein A2 (FOXA2). On day 20, they were tested for the other markers. Cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.1% TrionX-100, and blocked with 10% goat serum (Sigma-Aldrich) for 1 h at room temperature. Then the cells were incubated overnight with primary antibodies at 4 ℃. The primary antibodies against rat FOXA2 (1:200, Chemicon/Millipore, Billerica, MA, United States), SOX17 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, United States), albumin (ALB, 1:500), α-fetoprotein (AFP, 1:200), α1-antitrypsin (AAT, 1:200), cytokeratin 18 (CK18, 1:200, all from Santa Cruz Biotechnology), cytochrome P450 1A2 (CYP1A2, 1:200, Abcam, La Jolla, CA, United States) and cytochrome P450 3A4 (CYP3A4, 1:200, Abcam) were obtained. Then the cells were incubated with TRITC-conjugated secondary antibody (Invitrogen, Carlsbad, CA, United States) for 1 h at room temperature. The nuclei were then counterstained with 4,6-diamidino-2-phenylindole (DAPI, Roche, Mannheim, Germany). The cells were analyzed using a fluorescence microscope (IX71, Olympus, Japan).

Reverse-transcription PCR analysis of gene expression

To assess endodermal and hepatic markers, differentiated cells were tested for FOXA2 and SOX17 on day 5 and for other markers on day 20. Trizol reagent was used to extract total RNA from differentiated iPSCs (Invitrogen). Reverse transcription-PCR was used to produce cDNA. GenBank accession numbers, primer sequences, and amplicon size are listed in Table 1 for each gene. The PCR procedure was as follows: cDNA denaturation at 94 ℃ for 5 min followed by 28 cycles (ALB, 40 cycles) of denaturation at 94 ℃ for 15 s, annealing at 66 ℃ for 10 s, and extension at 72 ℃ for 20 s. The final extension took place at 72 ℃ and lasted 7 min. PCR products were separated by electrophoresis on agarose gels. The GAPDH housekeeping gene was used as an internal control.

Urea assay

The urea production test was performed using a colorimetric QuantiChrom Urea Assay Kit (BioAssay Systems, Hayward, CA, United States). Undifferentiated cells and differentiated cells on days 5, 10, 15, and 20 were trypsinized and counted with a hemocytometer. The assay was performed in accordance with the manufacturer's instructions. The sample supernatants were stored at -20 ℃. Absorbance was measured using a microplate reader. Urea production was normalized to the total number of cells.

Albumin secretion

The concentration of rat albumin in the supernatant was determined using a Rat Albumin ELISA Quantitation Kit (ICL, Portland, OR, United States) in accordance with the manufacturer's instructions. Undifferentiated iPSCs and differentiated cells were trypsinized and counted on days 5, 10, 15, and 20 using a hemocytometer. Albumin secretion was normalized to the total number of cells.

Periodic acid-Schiff staining

On day 20, differentiated iPSCs were stained using a Periodic acid-Schiff (PAS) staining system (Sigma-Aldrich). The cells were first fixed in 4% paraformaldehyde for 20 min and oxidized with 1% periodic acid solution for 5 min. Then they were washed. The cells were treated with Schiff's reagent for 15 min at room temperature. The cells were washed with PBS and then stained with hematoxylin for 90 s. They were then examined under a light microscope (IX50, Olympus, Japan).

Uptake of indocyanine green and low-density lipoprotein

After differentiation, cells were incubated for 1 h with indocyanine green (ICG, Sigma-Aldrich) in basal

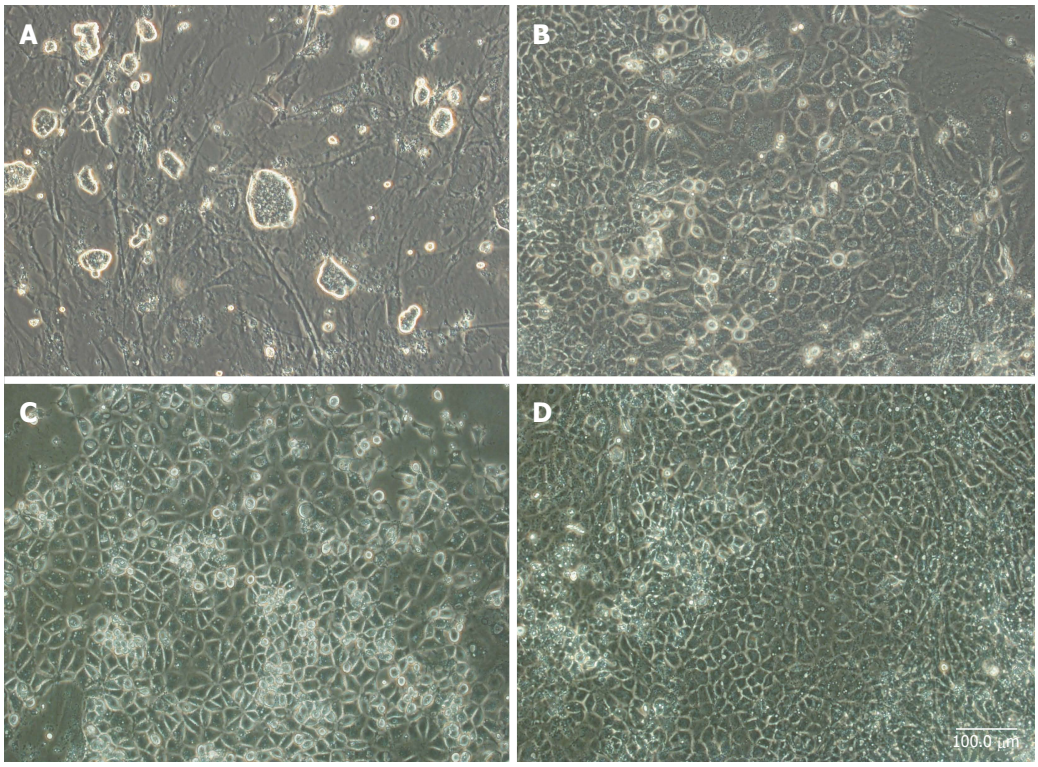


Figure 1 Morphological changes of rat induced pluripotent stem cells at different stages of differentiation. A: Undifferentiated induced pluripotent stem cells; B: Differentiated cells on day 5; C: Differentiated cells on day 15; D: Differentiated cells on day 20. Scale bar = 100 μm.

medium at 37 ℃. Uptake of ICG was detected under a light microscope. The ability of these cells to take up low-density lipoprotein was determined using an LDL Uptake Cell-Based Assay Kit (Cayman Chemical, Ann Arbor, MI, United States) according to the manufacturer's instructions. The cells were visualized under a fluorescent microscope.

Statistical analysis

Results are shown as mean ± SD. One-way ANOVA was used for statistical analysis. $P < 0.05$ was considered significant.

RESULTS

Morphological and phenotypic hepatic markers

A simple three-stage differentiation method for iPSCs was developed. During the first five days, iPSCs were treated in a medium with Activin A and Wnt3a for endodermal induction. The cells gradually took on flatter and spikier shapes (Figure 1B). Then, the cells were cultured under feeder-free conditions with high concentrations of FGF4 and BMP2 for the next five days. Cells in these colonies had compact, polygonal shapes like those of differentiated hepatocytes (Figure 1C). The medium was replaced with mature medium supplemented with HGF, OSM, and Dex for an additional 10 d. The cells exhibited the morphology of mature hepatocytes with large amounts of cytoplasm and distinct, round nuclei (Figure 1D).

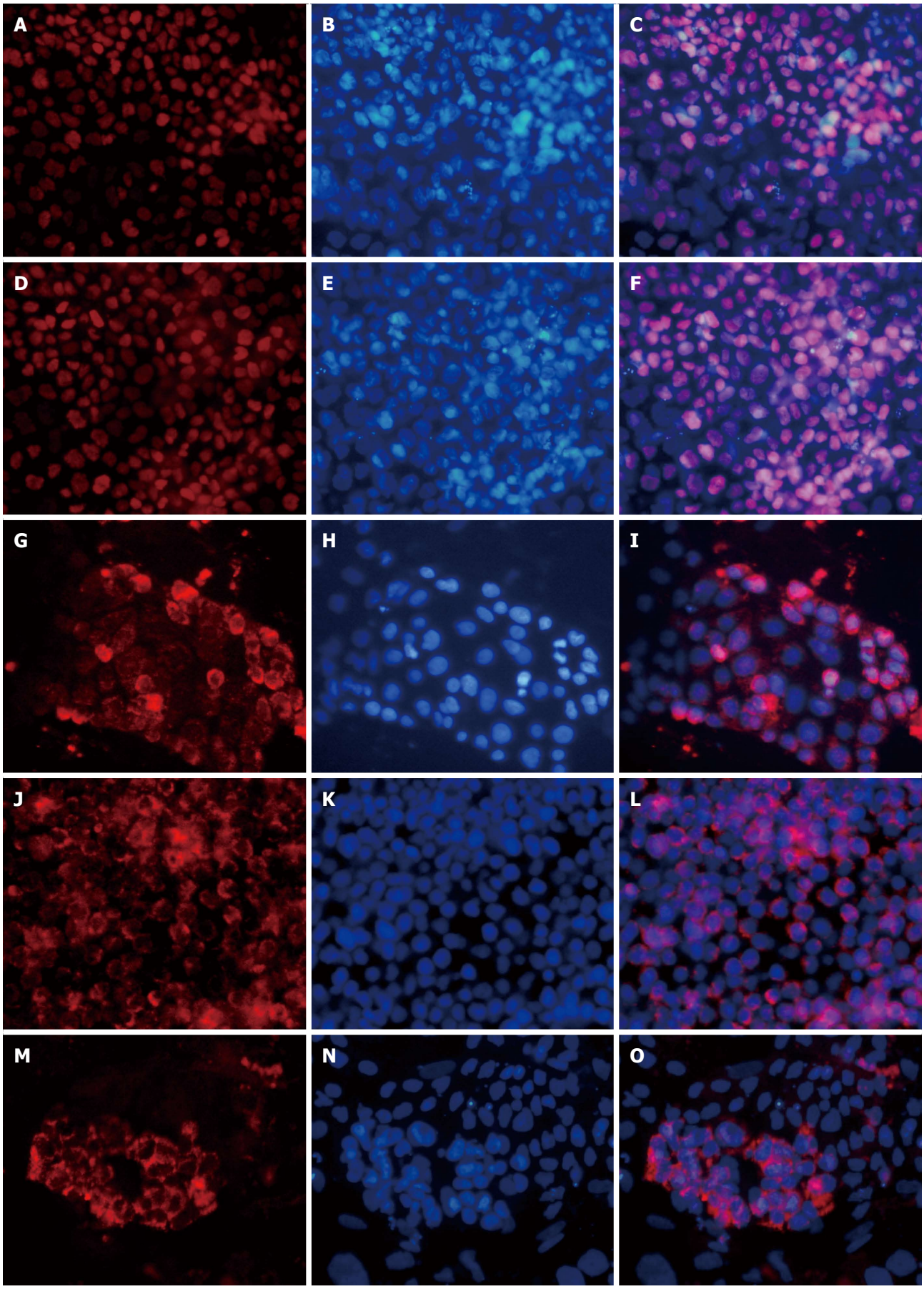
Cell characteristics were determined at different stages of differentiation using immunofluorescence staining. After endodermal induction, approximately 70%-80% of the differentiated cells expressed endodermal markers FOXA2 and SOX17 at the protein level (Figure 2). These results indicate that rat iPSCs could form endoderm efficiently if exposed to Activin A and Wnt3a. After hepatic maturation at stage 3, immunostaining analysis showed that 50%-60% of the cells expressed hepatic markers, including AFP, ALB, CK18, and functional proteins such as AAT, CYP1A2 and CYP 3A4 (Figure 2).

Gene expression profiles of HLCs

To further analyze the properties of iPSCs that developed into endodermal cells and HLCs, RT-PCR analysis was performed in the cells at various stages of differentiation. After induction of endodermal differentiation, endoderm markers FOXA2 and SOX17 were detected in the cells at stage 1 (Figure 3). This was consistent with the results of the immunostaining assay. After the cells differentiated into HLCs at stage 3, RT-PCR analysis indicated the expression of liver associated genes such as AFP, ALB, CK8, CK18, CK19, and transcription factor HNF-4α (Figure 3). These results collectively indicate that the cells had changed from endodermal to hepatic.

Functional characteristics of rat iPSC-derived HLCs

In order to evaluate the liver-specific function of iPSC-derived differentiated cells, the functional properties of HLCs of urea synthesis and albumin secretion were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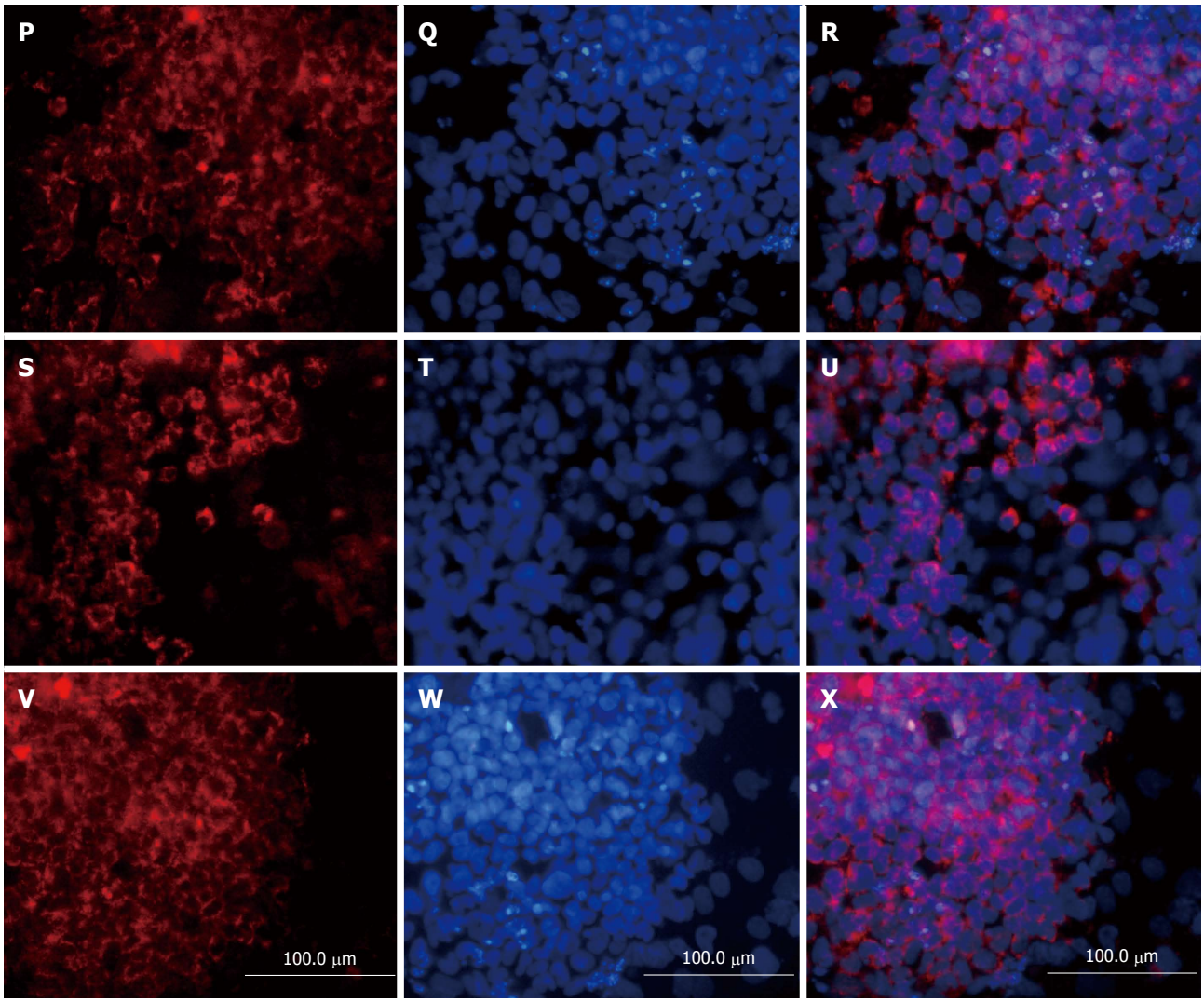

Figure 2 Protein expression in differentiated induced pluripotent stem cells on days 5 and 20 as indicated by immunofluorescence staining. A, B, C: FOXA2; D, E, F: SOX17; G, H, I: AFP; J, K, L: ALB; M, N, O: CYP1A2; P, Q, R: CYP3A4; S, T, U: AAT; V, W, X: CK18. Nuclei were stained blue with DAPI. Scale bar = 100 μm. FOXA2: Forkhead box protein A2; SOX17: SRY-box containing gene 17; AFP: α-fetoprotein; ALB: Albumin; CYP1A2: Cytochrome P450 1A2; AAT: α1-antitrypsin.

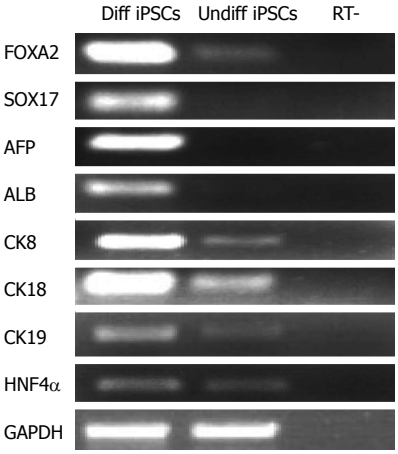

Figure 3 RT-PCR analysis of differentiated induced pluripotent stem cells on days 5 and 20. Differentiated cells expressed endodermal markers FOXA2 and SOX17 after 5 d of endoderm induction. Liver associated genes AFP, ALB, CK8, CK18, CK19, and HNF-4α mRNA were expressed on day 20. H2O served as a negative control. FOXA2: Forkhead box protein A2; SOX17: SRY-box containing gene 17; AFP: α-fetoprotein; ALB: Albumin; iPSCs: Induced pluripotent stem cells.

analyzed. The rates of urea synthesis (20 d 1.202 ± 0.080 mg/dL *vs* 0 d 0.317 ± 0.021 mg/dL, $P < 0.01$; Figure 4A) and albumin secretion (20 d 1.601 ± 0.102 mg/dL *vs* 0 d 0.313 ± 0.015 mg/dL, $P < 0.01$; Figure 4B) increased significantly as differentiation progressed, indicating hepatic maturation.

The ability of the cells to store glycogen was tested. PAS staining was used to confirm the presence of stored glycogen. Differentiated cells showed glycogen staining, indicating glycogen accumulation (Figure 4C).

The iPSCs-HLCs were also found to take up ICG and LDL (Figure 4D and E). These results showed that the iPSC-HLCs had the same function as hepatocytes *in vitro*.

DISCUSSION

iPSCs have been used to investigate hepatocyte differentiation and possible stem cell therapies. Studies have shown that human and mouse iPSCs can be

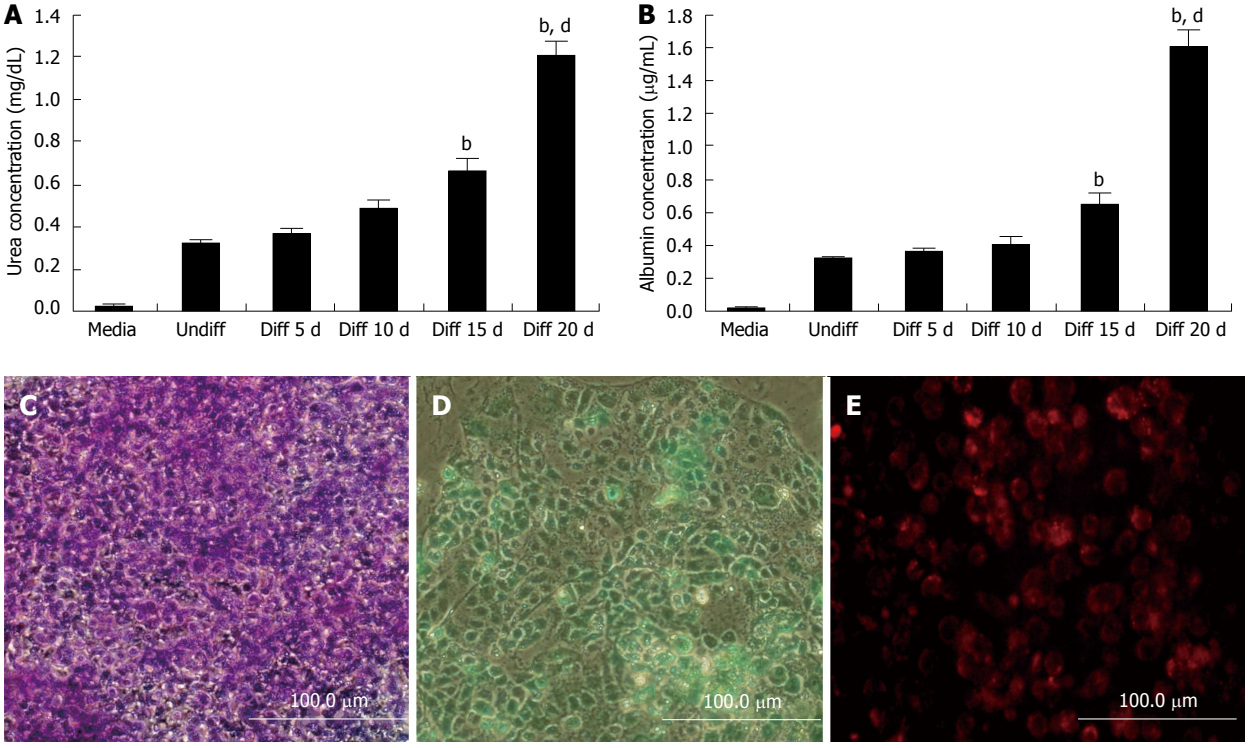

Figure 4 Functional analysis of differentiated induced pluripotent stem cells. A: Urea production of differentiated induced pluripotent stem cells (iPSCs); B: Albumin secretion of differentiated iPSCs; C: Glycogen synthesis of differentiated iPSCs as indicated by PAS staining on day 20; D: ICG uptake test of differentiated iPSCs; E: LDL uptake test of differentiated iPSCs. Scale bar = 100 μm. $^{b}P < 0.01$ *vs* undifferentiated cells; $^{d}P < 0.01$ *vs* differentiated cells on day 15.

differentiated into functional hepatocytes[20,21]. Currently, some of the studies of liver disease and possible therapeutic strategies involve animal models. Most models of liver diseases are based on rodents, especially rats. The present work is the first to demonstrate the efficient generation of HLCs differentiated from iPSCs of rat species. These differentiated cells were found to express the hepatic markers AFP, ALB, CK8, CK18, and CK19 and transcription factor HNF-4α. They were also able to store glycogen, secrete urea and albumin, and take up LDL and ICG. Results showed that these differentiated cells had a gene profile similar to that of HLCs derived from human and mouse iPSCs, as reported previously[20,24].

In the present study, a three-step protocol designed to generate HLCs from rat iPSCs was developed. The process is very simple and highly efficient. It takes two to three weeks and requires only a few inductive factors. The microenvironment in which the hepatocytes develop involves several biological events. Cell growth and differentiation are tightly regulated by a series of intra- and intercellular signals. All the inductive factors used in the process are extracellular signals[24]. As in previous studies, nodal signaling through the transforming growth factor-beta pathway was found to play a crucial role in the development of the endoderm. Activin A initiated this nodal pathway, causing the phosphorylation of Smad2 and Smad3. It also interacted with Smad4 and then activated gene-specific transcription factors in the nucleus[25]. Several studies have demonstrated that Activin A can be used during the formation of definitive endoderm. Hay and colleagues were able to enhance the endodermal differentiation of ESCs using Wnt3a in combination with Activin A. They postulated that Activin A plus Wnt3a would induce the rapid formation of a relatively consistent hepatic endoderm and cells that are more capable of acting like hepatocytes than other combinations of inductive factors[26]. In the present differentiation system, the iPSCs quickly became spiky in shape, and the formation of endoderm was induced using Activin A combined with Wnt3a, as previously reported[22,27].

FGFs and BMPs were found to play significant roles in liver specification. FGFs from the cardiac mesoderm and BMPs from mesenchymal cells of the septum transversum have been shown to be essential for the induction of foregut endoderm cells into hepatocytes[28]. Cai *et al*[29] found that both FGF4 alone and BMP2 alone in the culture medium had little effect on definitive endoderm cells into liver cells, but both together led to a pronounced increase in the number of cells expressing ALB *in vitro*. They also demonstrated that the induction of definitive endoderm is essential for the effectiveness of FGF4 and BMP2 in hepatic induction[29]. Another study showed that FGF4 plus BMP2 can generate hepatic cells from human iPSCs[20]. These factors were used in the present protocol at stage 2 of differentiation. Cells were driven toward commitment to hepatic cells.

To generate mature liver cells, the iPSCs were subjected to a process meant to foster *in vitro* maturation. At this stage, cells are generally grown in media that had previously been optimized for the culture of hepatocytes with the addition of factors thought to promote the maturation or proliferation of hepatocytes during liver development. OSM has been reported to facilitate the differentiation of liver progenitor cells into hepatocytes[30]. HGF enhances hepatic maturation and proliferation[27]. Dex is a synthetic glucocorticoid hormone. It is active in induction of enzymes involved in gluconeogenesis[24]. In the present work, during the part of maturation that involves these factors, differentiated cells presented a cuboidal morphology and showed the properties of hepatic cells.

The enzymes CYP1A2 and CYP3A4 are critical to drug metabolism. The present study showed that rat iPSC-derived HLCs expressed the hepatocyte function-specific proteins CYP1A2 and CYP3A4. This suggested that these cells gain liver-specific properties and may be used in drug metabolism tests and toxicity screens.

In summary, the data collected here suggest that rat iPSCs can differentiate into HLCs in a rapid and efficient manner through a three-stage process involving the application of inductive factors. Differentiated cells may be an attractive resource for treatment of end-stage liver disease.

COMMENTS

Background

Recent studies have suggested that differentiated hepatocytes derived from transplanted stem cells may be a suitable form of therapy. Embryonic stem cells (ESCs) and bone marrow-derived liver stem cells can form mature hepatocytes and reduce the severity of hepatic fibrosis. However, the ethical issues of ESCs and the low purity of bone marrow stem cells limit their use in clinical settings. Induced pluripotent stem cells (iPSCs) sidestep most of the ethical issues surrounding ESCs. The differentiation of iPSCs into hepatic lineages has been observed in humans and mice. So far, no reports have been published on the differentiation of rat iPSCs into hepatocyte-like cells (HLCs).

Research frontiers

The laboratory rat was the first mammalian species domesticated for scientific research, and the rat model is commonly used in research involving hepatic diseases. This study is the first to show the efficient generation of HLCs differentiated from the iPSCs of rats.

Innovations and breakthroughs

Hepatic differentiation was achieved using a three-step protocol. Rat iPSCs were differentiated into HLCs after 20 d of culture. These differentiated cells expressed hepatic markers and exhibited functional hepatic characteristics.

Applications

This study suggests that rat iPSCs can differentiate into HLCs in a rapid and efficient manner through a three-stage process involving the application of inductive factors. Differentiated cells may be an attractive resource for treatment of end-stage liver disease.

Terminology

Induced pluripotent stem cells are a type of pluripotent stem cell that can be generated directly from adult cells. Pluripotent stem cells hold great promise in the field of regenerative medicine. Because they can propagate indefinitely, as well as give rise to every other cell type in the body, they represent a single source of cells that could be used to replace those lost to damage or disease.

Peer-review

The manuscript is the first to demonstrate the efficient generation of HLCs differentiated from iPSCs of rat species.

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ORIGINAL ARTICLE

Basic Study

Effect of fibulin-5 on adhesion, migration and invasion of hepatocellular carcinoma cells *via* an integrin-dependent mechanism

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Author contributions: Tang JC carried out the experiments and drafted the manuscript; Liu JH and Liu XL participated in the experiments; Liang X analysed the data; Cai XJ designed the study and directed its implementation; all authors drafted the article and made critical revisions related to the intellectual content of the manuscript, and approved the final version of the article to be published.

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Institutional review board statement: All samples from the patients were taken after informed consent and ethical permission were obtained for participation in the study.

Conflict-of-interest statement: To the best of our knowledge, all the listed authors have participated actively in the study, and have seen and approved the submitted manuscript. The authors do not have any possible conflicts of interest.

Data sharing statement: We confirm that no additional unpublished data are available.

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Abstract

AIM: To elucidate the role of fibulin-5 (FBLN-5) as a suppressor of hepatocellular carcinoma (HCC) cell metastasis *via* integrin.

METHODS: The expression of FBLN-5 was determined by immunohistochemistry in 140 HCC samples and matched normal tissues, and was further confirmed by RT-PCR and Western blot analyses in various cell lines. Recombinant FBLN-5 was expressed in *Escherichia coli* BL21(DE3), purified and used in cell attachment assays. Expression of a specific plasmid or a specific siRNA in HCC cells resulted in the overexpression or knockdown of FBLN-5, respectively. Further, the migration and invasion of HCC cells were investigated using the Boyden chamber and transwell assays. The concentration of secreted matrix metalloproteinase 7 (MMP-7) was determined using ELISA.

RESULTS: FBLN-5 expression was found to be downregulated in HCC. Its expression was significantly correlated with advanced tumor metastasis; this was indicative of poor 5-year overall survival. Recombinant full-length human FBLN-5 promoted the attachment of HCC cells *via* integrins: it inhibited HCC cell adhesion

and migration to fibronectin in a concentration-dependent manner. It also inhibited HCC cell migration and invasion through an integrin-binding arginine-glycine-aspartic acid (RGD) motif by downregulating MMP-7.

CONCLUSION: These results suggest that lower FBLN-5 expression is an important indicator of poor survival and that FBLN-5 inhibits HCC motility *via* an integrin-dependent mechanism. RGD-dependent suppression of MMP-7 by FBLN-5 might contribute to the development of new therapeutic strategies for HCC.

Key words: Fibulin-5; Hepatocellular carcinoma; Integrin; Adhesion; Migration; Invasion

Core tip: Fibulin-5 (FBLN-5) is a matricellular protein that contains an arginine-glycine-aspartic acid motif, the role of which is to bind certain integrins and thereby mediate cancer cell motility. Several studies have revealed that FBLN-5 may promote or suppress tumor progression through its interaction with integrins in various human tumors in a context-specific manner, which might be a crucial event in the invasiveness of malignant tumor cells.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, and it is associated with a considerably high mortality rate. At present, more than 0.7 million people have been diagnosed with HCC, which corresponds to an incidence of 16 per 0.1 million people[1]. In 90% of the cases, HCC is a result of chronic liver damage and is therefore a typical inflammation-related cancer characterized by the close association between the tumor microenvironment and tumor cells.

Changes in the interaction between the extracellular matrix and tumor cells play an important role in tumorigenesis and metastasis. Fibulin-5 (FBLN-5) is an extracellular matrix glycoprotein that has been shown to be expressed in elastin-rich tissues; it mediates changes in the matrix structures *via* its interaction with several extracellular proteins such as tropoelastin, elastin monomers, lysyl oxidase-like proteins, and latent TGF-β-binding proteins[2]. FBLN-5 contains an arginine-glycine-aspartic acid (RGD) motif, the function of which is to bind to certain integrins and mediate the adhesion of endothelial cells[3,4]. Moreover, FBLN-5 also inhibits the expression of matrix metalloproteinase 7 (MMP-7) in lung cancer cells *via* an integrindependent mechanism and downregulation of the ERK pathway[5]. Experiments on FBLN-5-null mice have provided evidence for the role of FBLN-5 as an angiogenesis inhibitor and its roles in the proliferation, migration and invasion of certain tumors. The effect of FBLN-5 on tumorigenesis appears to be largely context-dependent, and may involve the inhibitory effect of fibulin-5 on angiogenesis; however, the exact mechanisms are still under investigation[2].

In this study, we investigated the expression of FBLN-5 and its relationship with clinicopathological features in order to understand its role in HCC progression, and to identify the molecular mechanisms responsible for its functions.

MATERIALS AND METHODS

Plasmids

Site-directed mutagenesis of eukaryotically expressed FBLN-5 was performed, wherein Asp56 was substituted with Glu within the integrin-binding RGD motif, so as to prevent integrin binding[6,7]. The KOD Hot start DNA polymerase kit (Millpore, United States) was used according to the manufacturer's recommendations.

The primers that were used for site-directed mutation PCR were: forward, 5′-TCC CCG AGG CCT GCC GAG GAG AAA TGA TGT GTG TTA ACC AAA ATG-3′ and reverse, 5′-CAT TTT GGT TAA CAC ACA TCA TTT CTC CTC GGC AGG CCT CGG GGA-3′.

Tissue samples

One hundred and forty HCC samples and normal tissue samples were collected from patients with HCC (115 males and 25 females) who underwent surgery at Sir Run Run Shaw Hospital of Zhejiang University between January 2006 and December 2010. The procedures and data collection were approved by the human research committee of Sir Run Run Shaw Hospital, and written informed consent was obtained from all patients before the study was started. Clinicopathological data including age, gender, tumor size, nodal status, physiological and biochemical indicators, Barcelona clinic liver cancer (BCLC) tumor stage, and tumor-node-metastasis (TNM) stage were obtained retrospectively from the patients' clinical records and pathology reports. Before the surgery was performed, the patients underwent staging examinations, including blood routine examination, liver function tests, abdominal ultrasonography and/or magnetic resonance imaging. Based on the Union for International Cancer Control (UICC) 1987 and BCLC system, 18 patients were found to have stage Ⅰ cancer; 84, stage Ⅱ; 30, stage Ⅲ; and 8, stage Ⅳ.

Based on the BCLC tumor staging system alone, 14 patients were found to have stage 0 cancer; 79, stage A; 28, stage B; 16, stage C; and 3, stage D. The survival rates were determined based on the data from the cancer registry of our hospital, or the required information was collected from the patients' attending physicians.

Immunohistochemistry

Normal and HCC tissue samples were selected based on the diagnosis and microscopic observation of the tumor tissue. Immunohistochemistry was performed with mouse antibodies against FBLN-5 (Abcam, United Kingdom) as previously described[8].

The immunostaining results were examined independently by two pathologists who were blinded to the clinical data. FBLN-5 staining was scored from 0 to +3: 0 indicated that none of the cells were stained; +1, less than 5% of the cells were stained; +2, 5%-50% of the cells were stained; and +3, more than 50% of the cells were stained. Further, the intensity of staining was graded from 0 to 3 as previously described[9].

Semi-quantitative real-time PCR assays and methylation-specific PCR

PCR amplification of FBLN-5 mRNA (forward: 5′-CCAAACTATCCCACGATC-3′; reverse: 5′-CAGGAACATTCGCACAG-3′) and GAPDH mRNA (forward: 5′-ACAGTCAGCCGCATCTTCTT-3′; reverse: 5′-TGGAAGATGGTGATGGGATT-3′) was performed using SsoFast EvaGreen Supermix with the Low ROX Kit (BioRad, United States), as previously described[10]. For methylation-specific PCR (MSP), the methylated FBLN-5 promoter was amplified using the primers reported previously[5]. MSP products were analyzed by electrophoresis on 2% agarose gels.

SDS-PAGE and Western blot analysis

Cell samples were homogenized in RIPA lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% NP-40, 0.5% Na-deoxycholate, and 0.1% SDS] for protein extraction. Western blot analysis was performed as described in a previous study[10]. Antibodies against V5 (Cell Signaling Technology, United States), FBLN-5 (Abcam, United Kingdom), MMP-7 (Cell Signaling Technology, United States) and GAPDH (Sigma, Germany) were used. The blots were visualized using Image Quant LAS-4000 (Fujifilm, Japan).

Transgene expression and recombinant FBLN-5 production

We used four cell lines: the human immortalized normal hepatocyte cell line LO2 and three HCC cell lines (MHCC97L, MHCC97H and HCC-LM3). These cell lines were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China. The cells were maintained in complete Dulbecco's modified Eagle medium (DMEM, Gibco, United States). Two other HCC cell lines (HepG2 and Hep3B) obtained from American Type Culture Collection (ATCC, United States) were cultured in minimum essential medium (MEM, Gibco, United States). All the media contained 10% (v/v) fetal bovine serum (FBS; Gibco, United States) and 1% (v/v) penicillin/streptomycin. The cells were incubated at 37 ℃ in a humidified atmosphere containing 5% CO_2.

The plasmids (pcDNA3.1-FBLN-5, pcDNA3.1-FBLN-5 with the RGD mutation, and pcDNA3.1) or small interfering RNA (siRNA against FBLN-5) was transiently transfected into cells with Lipofectamine 2000 or the RNAi MAX reagent according to the manufacturer's instructions (Invitrogen, United States).

Proteins fused with glutathione *S*-transferase (GST) in the pGEX vector were expressed in *Escherichia coli* BL21(DE3), induced with 1 mmol/L isopropyl-α-D-thiogalactoside (IPTG; Promega, United States), and purified according to the manufacturer's instructions for glutathione sepharose beads TM 4B (GE, United States). Purification experiments for the GST fusion protein were carried out as previously described[11]. Unless otherwise stated, FBLN-5 expressed by the prokaryotic system represented the recombinant protein with GST and full-length FBLN-5, and prokaryotically expressed FBLN-5-RGE represented the recombinant protein with GST and full-length FBLN-5 D56E.

Analysis of secreted MMP-7

The concentration of secreted MMP-7 was determined in triplicate by ELISA using the Human Total MMP-7 Quantikine ELISA kit (R&D Systems, United States) according to the manufacturer's protocol.

Cell attachment assays

The synthetic peptides GRGDS and SDGRG (Sangon Biotech, China) were used to determine whether FBLN-5 binds to the HCC cells *via* integrin-binding to the RDG domain. Recombinant full-length FBLN-5 and FBLN-5-RGE were diluted to 20-400 nmol/L in $DPBS^+$ and incubated overnight in 96-well microplates at 4 ℃ in order to facilitate adsorption onto the wells. Non-specific binding was blocked using 10 mg/mL heat-denatured bovine serum albumin (BSA) for 1 h. BSA was aspirated, and 100 μL of HCC-LM3 or Hep3B cell suspension (5×10^5 cells/mL) was added to the wells and incubated for about 30 min at 37 ℃ in a 5% (v/v) CO_2 atmosphere. In order to determine the number of cells that had adhered, a known number of cells was added to the wells and fixed with 10 μL of 4% paraformaldehyde. Each well was washed three times with 200 μL of $DPBS^+$, and the cells were stained with 100 μL of 0.1% (w/v) crystal violet for 1 h. The dye that was taken up by cells was solubilized in 100 μL of

10% (v/v) acetic acid, and absorbance was measured at 590 nm. The absorbance of the cells present in the wells was considered as the percentage of cell attachment.

Cell migration assay

Each well was coated with 200 nmol/L of FBLN-5, FBLN-5-RGE or fibronectin (FN) in $DPBS^{+}$ overnight at 4 ℃. Then, the cells were incubated with 10 mg/mL heat-denatured BSA for 1 h at 20 ℃ to block non-specific binding. The effect of FBLN-5 on the migration of HCC cells and on the response of the cells to TGF-β was determined as follows: (1) the HCC-LM3 cells that had adhered to the wells were trypsinized, neutralized, seeded at confluence (5×10^5 cells/well), and incubated at 37 ℃ in a 5% CO_2 incubator for up to 12 h, until a confluent monolayer was formed. The cells were then serum-starved overnight and washed twice with serum-free DMEM before wounding with the tip of a sterile P10 pipette. The effect of FBLN-5 on the migration of HCC-LM3 cells was assessed at the time of wounding. After 48 h, the wound was photographed in order to determine the number of cells that had migrated into the cleared wound; and (2) cell migration was evaluated by counting the number of HCC-LM3 cells that had migrated on three independent membranes under a 100 × phase-contrast microscope; the number was then normalized using the number of the control cells (in transwell chambers coated with BSA) in order to determine the relative ratio. After the excess dye was washed out and dried, the crystal violet adsorbed by the cell nuclei was extracted with 10% acetic acid. Absorption of the destained cells at 590 nm was measured.

Cell invasion assay

Invasion assays were performed in 24-well transwell units with 8-μm filters coated with 1:6 diluted matrigel (BD Biosciences, United States); the assays were performed in triplicate. Each well contained approximately 2×10^6 cells. After 36 h of incubation, the cells that passed through the filters into the bottom wells were fixed in formalin and stained with crystal violet. The number of cells in 10 randomly selected fields (× 100) from each well was counted under a microscope.

Statistical analysis

Unless otherwise stated, data are expressed as mean ± SD and analyzed using the Student's *t*-test (GraphPad Prism 5.02); differences were considered significant when *P* value was less than 0.05.

RESULTS

Downregulation of FBLN-5 in HCC specimens and cells

To determine whether FBLN-5 plays a role in the pathogenesis of HCC, semi-quantitative reverse transcription-PCR (RT-PCR) and Western blot analyses were performed on three HCC and six normal tissue samples. FBLN-5 expression was low in the tumor specimens and high in the normal tissues (Figure 1A). Next, mRNA expression of FBLN-5 in another batch of 10 matched sets of frozen tumor and normal samples was analyzed using qRT-PCR; the results were found to be consistent with those of mRNA expression of FBLN-5 ($P < 0.05$) (Figure 1B).

The downregulation of FBLN-5 was further confirmed by qRT-PCR and Western blot in HCC cells including MHCC97L, MHCC97H, HCC-LM3, HepG2 and Hep3B cells, and cells of the immortalized normal hepatocyte cell line LO2 ($P < 0.05$, Figure 1C and D). Moreover, to investigate whether the downregulation of FBLN-5 is due to epigenetic silencing, the above-mentioned 10 matched tissue samples were analyzed using the MSP assay, and methylation of the FBLN-5 promoter was not found in any of the tumor samples (0%). Similar phenomena were also observed in HCC-LM3, MHCC97H, MHCC97L, HepG2 and Hep3B cells (Figure S1). Collectively, these findings indicate that FBLN-5 expression is downregulated in both HCC tissues and cells, and suggest that FBLN-5 might participate in the pathogenesis of HCC.

Clinicopathological features and survival of HCC patients according to FBLN-5 expression

To investigate whether downregulation of FBLN-5 expression is associated with certain prognostic factors, we classified the patients into groups based on their immunohistochemistry findings. As shown in Table 1, patients with a higher number of tumor nodules ($n \geqslant 2$) and BCLC stages B-D and TNM stages Ⅲ-Ⅳ tumor had significantly lower FBLN-5 expression than patients with a single tumor nodule ($P = 0.023$) and BCLC stages 0-A ($P = 0.001$) and TNM stages Ⅰ-Ⅱ ($P = 0.001$) tumor. No significant difference was found in the FBLN-5 level according to age, gender, tumor size, presence of HBV, serum AFP level, or histological type at the time of diagnosis.

Disease-specific survival analysis using Cox regression revealed that the prognosis of patients with low expression of FBLN-5 in the tumor cells was significantly poorer than that of patients with high expression of FBLN-5 ($P = 0.001$; Table 2). FBLN-5 expression was indeed associated with poorer overall survival and disease-free survival in HCC patients ($P < 0.05$, respectively; Figure 2). These results clearly indicate that FBLN-5 may act as a potent biomarker of prognosis of HCC patients.

Attachment of HCC cells to FBLN-5 via integrin binding

To investigate the mechanism of integrin regulation by FBLN-5, we tried to determine the functional role of its RGD motif. A site-mutated (D56E) RGD motif in pGEX-FBLN-5 was constructed and named FBLN-5-RGE (Figure 3A). GST, GST-FBLN-5 and GST-FBLN-5-

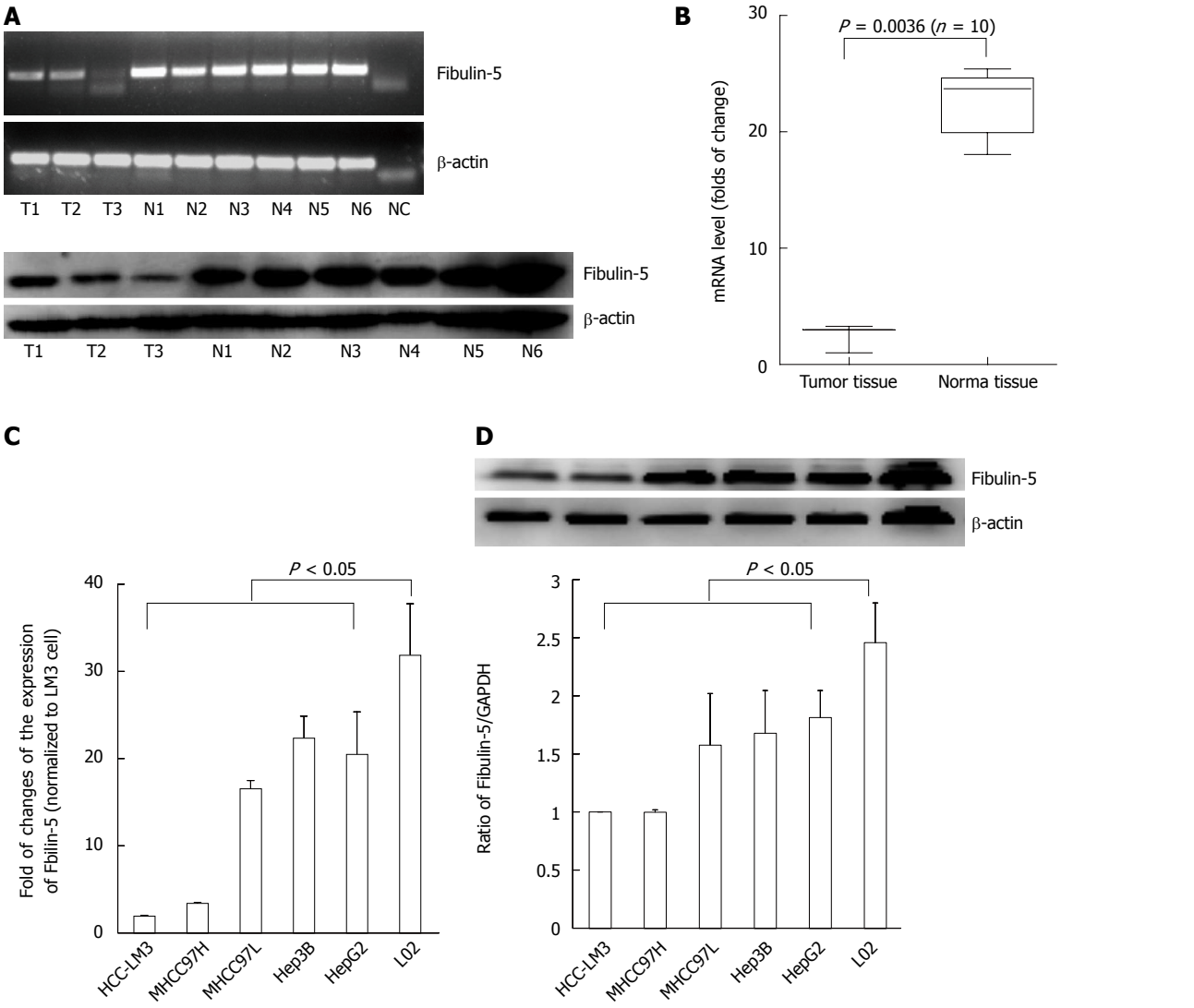


Figure 1 Decreased expression of fibulin-5 in hepatocellular carcinoma specimens. A: RT-PCR and Western blot analyses of Fibulin-5 (FBLN-5) expression in three hepatocellular carcinoma (HCC) samples (T) compared to that in six normal tissues (N). β-actin was used as an internal control; B: Q-RT-PCR analysis of FBLN-5 expression in ten HCC samples compared to that in matched tumor-adjacent tissues. Values are given as the mean ± SD and analyzed by Student's *t*-test; C: Comparing differences in the expression levels of FBLN-5 between HCC cell lines with different metastatic potentials and the immortalized hepatic cell line LO2 by Q-RT-PCR ($P < 0.05$ *vs* LO2, $n = 3$); D: Western blot analysis of FBLN-5 protein expression levels in HCC lines and LO2 cell line. The values of the band intensity below the figure represent the densitometric estimation of each band normalized by β-actin ($P < 0.05$ *vs* LO2, $n = 3$).

RGE were purified and pulled down using glutathione sepharose beads, and monitored using SDS/PAGE and Coomassie blue staining (Figure 3B). Since FN promotes integrin-mediated cell attachment *via* its RGD motif[12], in cell attachment assays, human plasma FN diluted to 10-400 nmol/L in DPBS$^+$ was used as the positive control. HCC cells in BSA-blocked wells were used as a negative control, and full-length FBLN-5 and FBLN-5-RGE were used in all the subsequent experiments.

Compared to FBLN-5, FBLN-5-RGE supported the adhesion of a much lower number of HCC-LM3 cells (Figure 3C; 35% at 200 nmol/L). We also found that Hep3B, another typical type of HCC cell, resulted in a higher percentage of attachment to FBLN-5 than to FBLN-5-RGE (Figure S2A; 65% and 40%, respectively, at 400 nmol/L). The findings revealed that the RGD motif was necessary for the binding of FBLN-5 to HCC cells. FBLN-5 or FN at 200 nmol/L in DPBS$^+$ and at 400 nmol/L resulted in the attachment of a similar number of HCC cells, with the former concentration showing minimal endotoxicity. According to the above results, the concentration of 200 nmol/L of FBLN-5 or FN in DPBS$^+$ was selected for further experiments, as this seems to be the minimum concentration necessary for the *in vitro* adhesion of HCC cells.

Moreover, pre-incubation of HCC cells with the synthetic peptide GRGDS resulted in significant block of FBLN-5 adhesion ($P < 0.001$ compared with untreated HCC-LM3 cells exposed to FBLN-5), while incubation with the control peptide SDGRG did not result in inhibition of cell attachment both in HCC-LM3 cells and Hep3B cells (Figure 3D, S2B). These results indicate that HCC cells bind to FBLN-5 *via* an integrin-

Table 1 Demographic features and clinicopathologic correlation of fibulin-5 in patients with hepatocellular carcinoma

Parameter		Total No. of patients (n = 140)	FBLN-5 expression		P value[1]
			High (n = 93)	Low (n = 47)	
Age (yr)	< 50	54	40	14	0.211
	⩾ 50	86	55	31	
Gender	Male	113	79	34	0.287
	Female	27	16	11	
Tumor size (cm)	< 5	82	57	25	0.618
	⩾ 5	58	38	20	
No. of tumor nodules	1	122	87	35	0.023[2]
	⩾ 2	18	8	10	
Recurrent Cancer	Absent	87	64	23	0.064
	Present	53	31	22	
Cirrhosis	Absent	50	29	21	0.063
	Present	90	66	24	
Serum AFP level	Negative	79	52	27	0.558
	Positive	61	43	18	
HBV	HBsAg (-)	32	22	10	0.902
	HBsAg (+)	108	73	35	
TNM tumor stage	Ⅰ + Ⅱ	102	79	23	0.001[2]
	Ⅲ + Ⅳ	38	16	22	
BCLC tumor stage	0 + A	93	74	19	0.001[2]
	B + C + D	47	21	26	

[1]P value from χ^2 or Fisher exact test; [2]Statistically significant. HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; AFP: Alpha-fetoprotein; TNM: Tumor-node-metastasis; HBsAg: Hepatitis B surface antigen; FBLN-5: Fibulin-5; BCLC: Barcelona clinic liver cancer.

Table 2 Disease-specific survival by Cox regression analysis between the subgroups of various clincopathological parameters

Parameter		No.	No. of events	HR (95%CI)[1]	P value[2]
Age (yr)	< 50	54	18	0.373 (0.169-0.823)	0.158
	⩾ 50	86	24		
Gender	Male	113	30	0.864 (0.324-2.306)	0.771
	Female	27	12		
Tumor size (cm)	< 5	82	23	1.874 (0.902-3.895)	0.093
	⩾ 5	58	19		
No. of tumor nodules	1	122	32	3.634 (1.481-8.916)	0.005[3]
	⩾ 2	18	10		
Recurrent cancer	Absent	87	15	0.587 (0.231-1.491)	0.263
	Present	53	28		
Cirrhosis	Absent	50	14	0.924 (0.426-2.004)	0.841
	Present	90	29		
Serum AFP level	Negative	79	21	0.615 (0.280-1.352)	0.227
	Positive	61	21		
HBV	HBsAg (-)	32	11	1.487 (0.670-3.302)	0.329
	HBsAg (+)	108	31		
TNM tumor stage	Ⅰ + Ⅱ	102	11	7.478 (2.571-21.753)	0.001[3]
	Ⅲ + Ⅳ	38	31		
BCLC tumor stage	0 + A	93	8	5.257 (1.707-16.186)	0.004[3]
	B + C + D	47	34		
FBLN-5 Expression	High	95	13	4.276 (2.033-8.995)	0.001[3]
	Low	45	29		

[1]The 95% Wald Confidence Limits; [2]Associations determined by Cox regression analyses; [3]Statistically significant. AFP: Alpha-fetoprotein; HBV: Hepatitis B virus; TNM: Tumor-node-metastasis; BCLC: Barcelona clinic liver cancer; FBLN-5: Fibulin-5; HBsAg: Hepatitis B surface antigen.

dependent mechanism.

Inhibition of FN-mediated HCC cell adhesion and migration by FBLN-5

FBLN-5 and FN both ligate integrin[13], so the possibility of FBLN-5 modulation of FN-mediated adhesion and migration of HCC-LM3 cells was investigated. To determine whether the adhesion of HCC-LM3 cells to FBLN-5 influences migration, 50000 HCC-LM3 cells were added to each chamber of the transwell, the bottom of which contained membranes coated with BSA or FBLN-5 and FN in a range of ratios (F5:FN). Compared to groups with high FN or high FN/FBLN-5 levels, the number of migrated HCC-LM3 cells was significantly lower in the groups with FBLN-5 or lower FN/FBLN-5 ratios (P < 0.001; Figure 4A). Moreover,

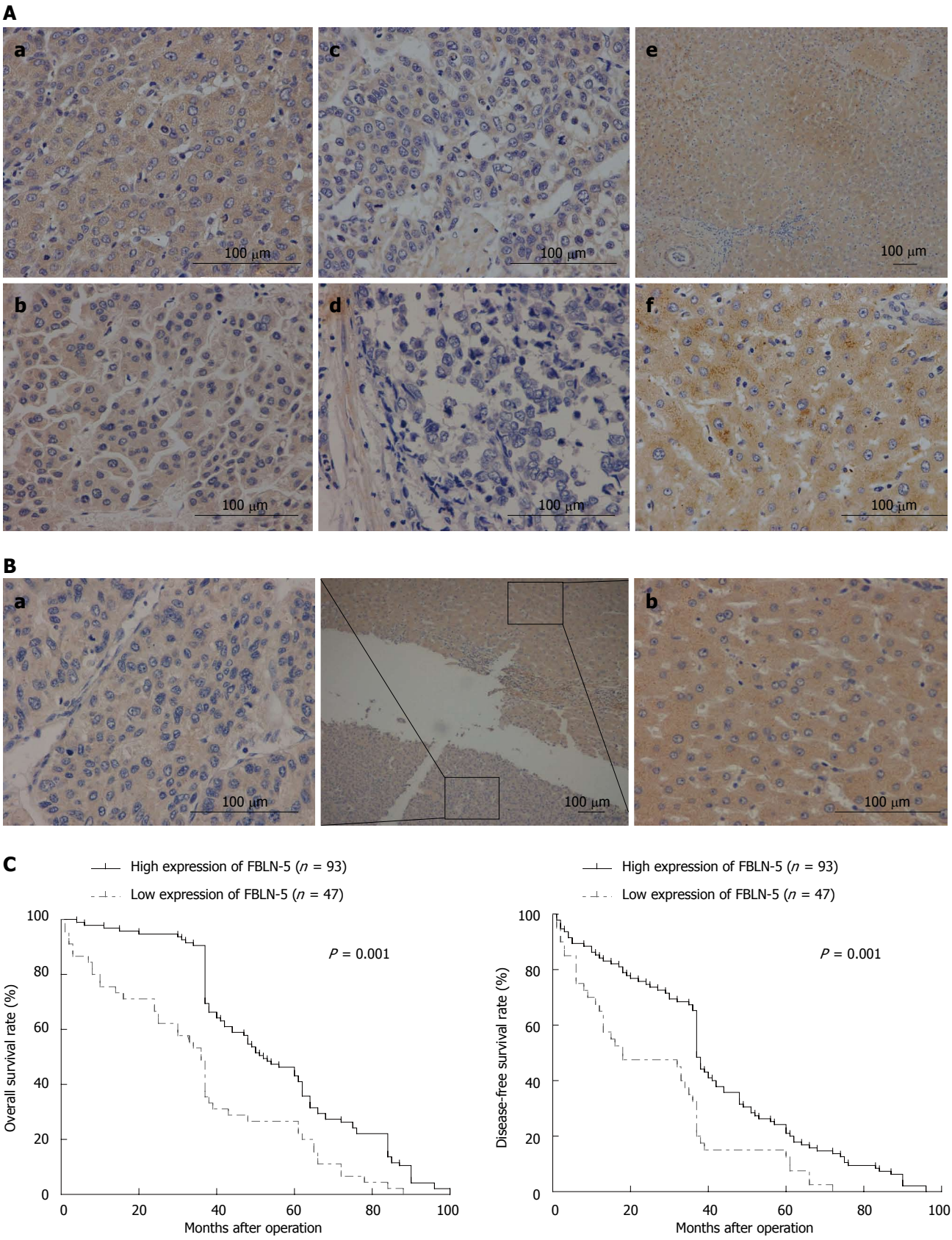


Figure 2 Immunohistochemical staining of fibulin-5 and disease-specific survival in patients with hepatocellular carcinoma. A: The intensity of fibulin-5 (FBLN-5) expression in various tissues by immunohistochemical staining was evaluated in tumor tissues of hepatocellular carcinoma (HCC) patients with a: Stage Ⅰ; b: Stage Ⅱ; c: Stage Ⅲ; d: Stage Ⅳ, and e-f: Normal tissue samples with various magnification. Normal control is fresh liver tissues from patients who underwent the resection for other non-HCC diseases; B: The intensity of FBLN-5 expression in a: tumor tissues; and b: pericarcinomatous tissue in same patients with HCC; C: Survival of patients with low FBLN-5 expression (dashed grey line) was significantly abbreviated in comparison to those with high expression (solid black line), with a statistically significant difference both in a: Overall (P = 0.001); and b: Disease-free survival (P = 0.002) by log-rank test.

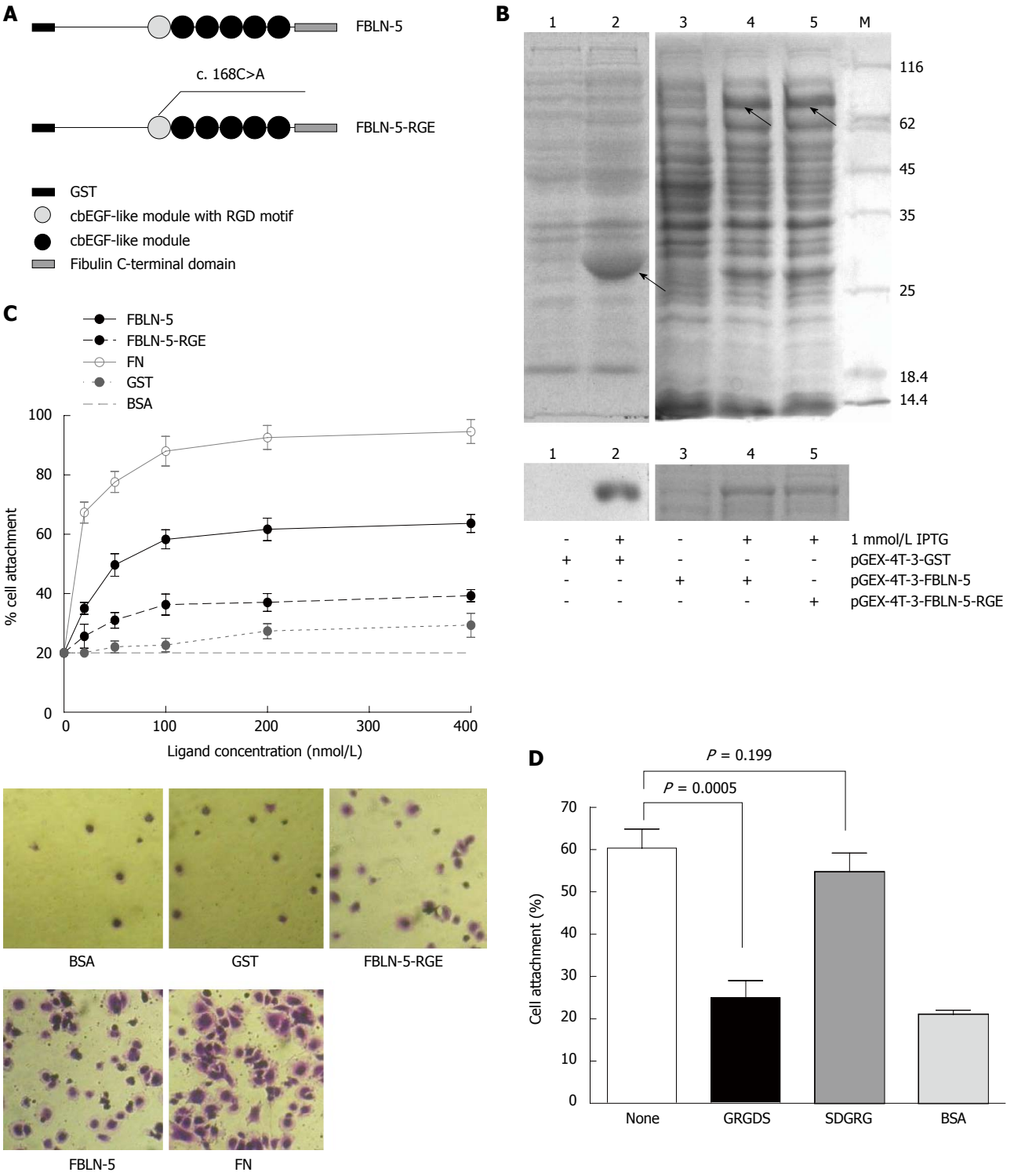


Figure 3 Characterization of recombinant human fibulin-5 and the attachment to hepatocellular carcinoma-LM3 cells. A: Domain organization of recombinant fusion protein of GST-FBLN-5 and GST-FBLN-5-RGE (cbEGF-like module, calcium binding EGF-like module); B: SDS/PAGE analysis of glutathionesepharose affinity-purified GST, recombinant full-length FBLN-5, and FBLN-5-RGE under non-reducing conditions. M indicates molecular-mass markers (sizes in kDa). IPTG (1 mmol/L) significantly induced the expression of recombinant proteins (upper), which were purified with glutathionesepharose beads later (bottom); C: Attachment of HCC-LM3 cells on GST (black dotted line), FBLN-5 (black solid line), FBLN-5-RGE (black dashed line), and human plasma FN (grey solid line). Background cell adhesion on BSA-coated wells is also shown as grey dashed lines (upper). A high percentage of HCC-LM3 cells showed spreading on full-length FBLN-5 and on FN at 200 nmol/L, but many fewer cells spread on FBLN-5-RGE, GST and BSA were visible (bottom). Results are mean ± SD of three experiments; D: Inhibition of HCC-LM3 attachment to 200 nmol/L FBLN-5 by synthetic GRGDS peptide (500 μg/mL). Synthetic SDGRG peptide (500 μg/mL) was used as a negative control. None indicates HCC-LM3 attachment to 200 nm FBLN-5 in the absence of peptide. BSA indicates HCC-LM3 attachment to BSA-blocked wells. Pre-incubation of HCC-LM3 with the synthetic peptide GRGDS significantly blocked adhesion to FBLN-5 ($P < 0.001$, compared with untreated HCC-LM3 on FBLN-5). Pre-incubation with the control peptide SDGRG showed no inhibition of cell attachment ($P = 0.199$, compared with untreated cells on FBLN-5).

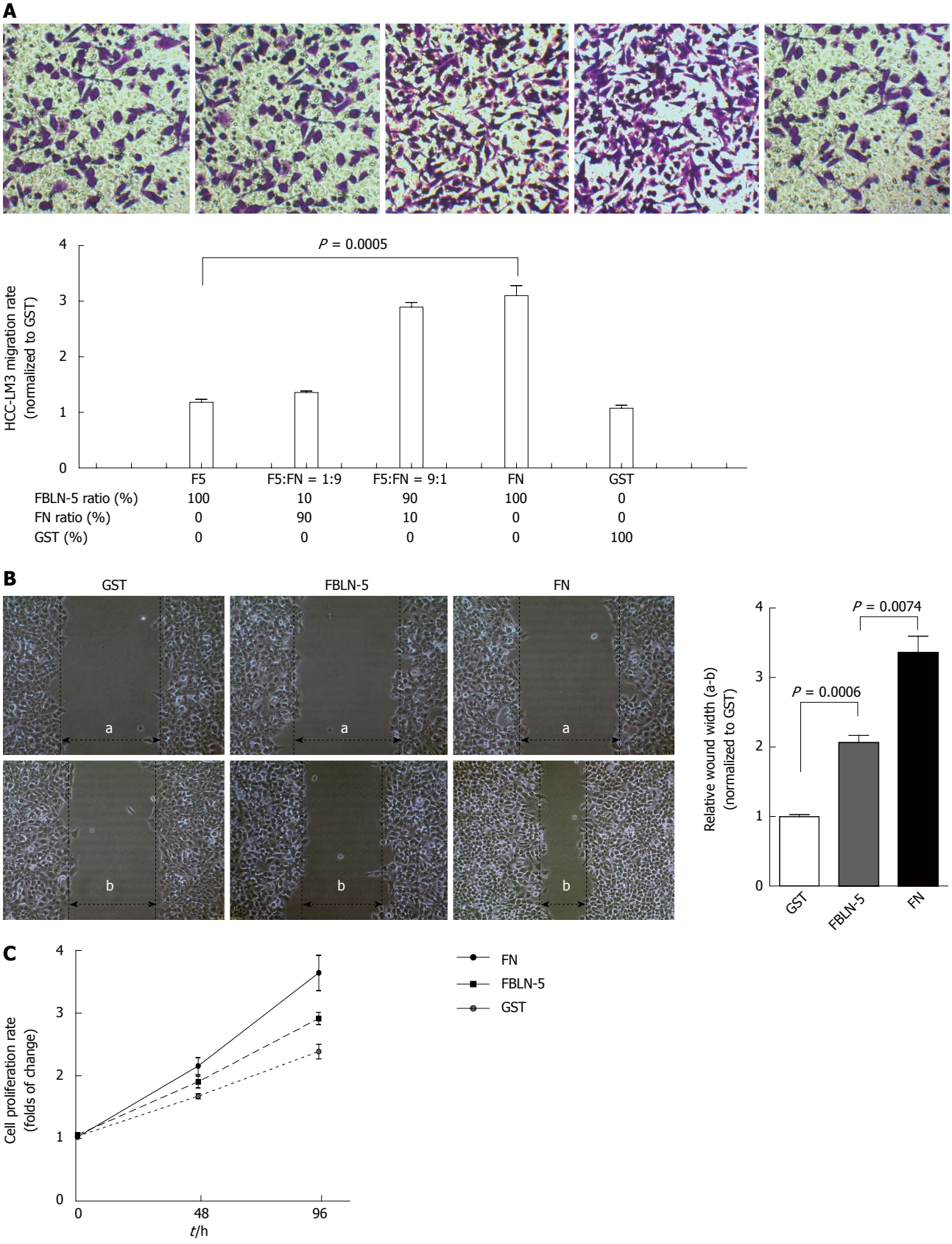
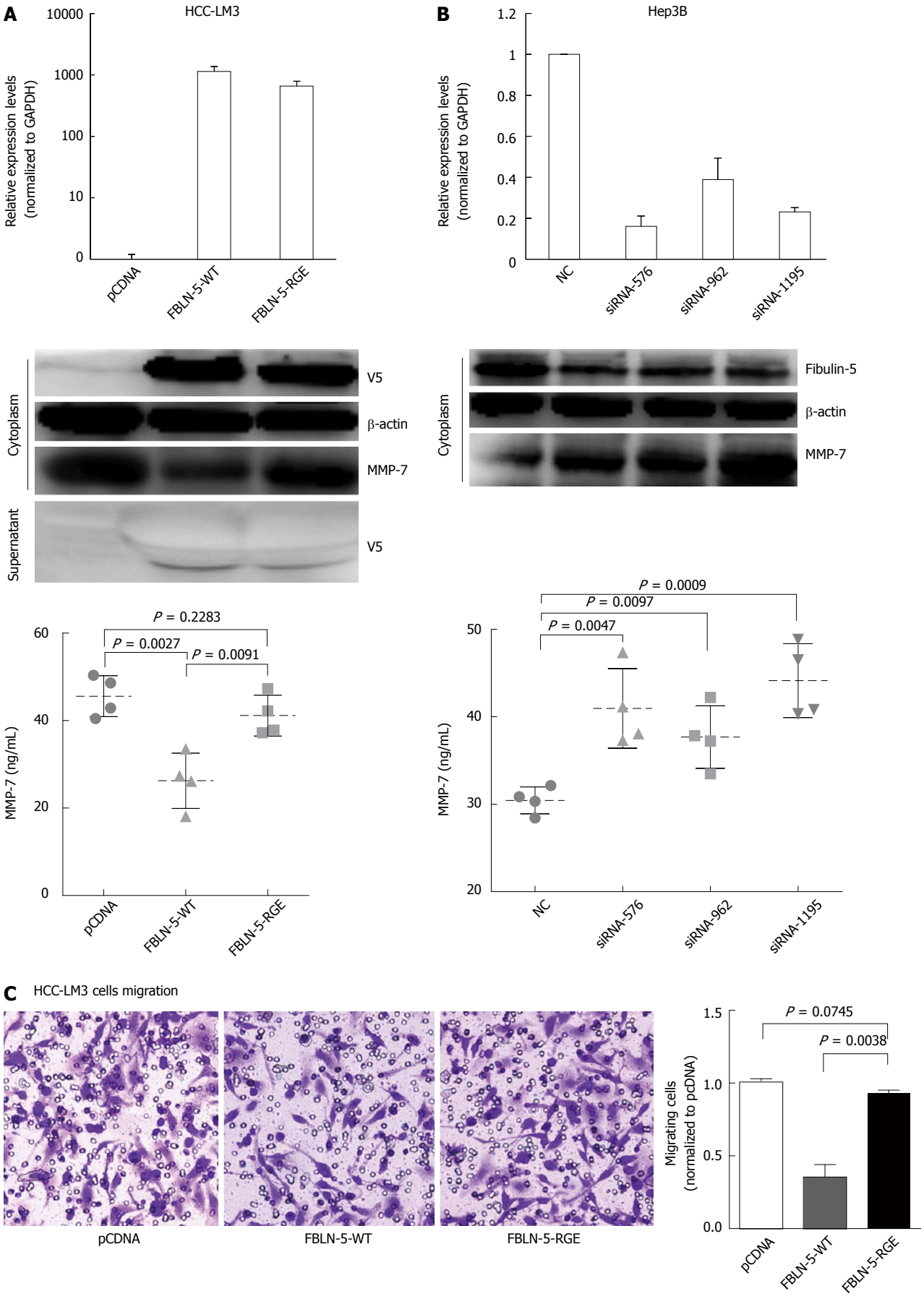


Figure 4 Hepatocellular carcinoma cell migration and proliferation on fibulin-5. A: The effects on hepatocellular carcinoma (HCC)-LM3 migrating of Boyden chamber and transwell assays at a range of fibulin-5 (FBLN-5)/FN ratios (F5:FN) as indicated were analysed. HCC-LM3 cell migration was inhibited by FBLN-5 as compared with FN group (upper). The histogram (bottom) represent the migration rate after calculating the absorption of the destained cells at 590 nm. Results are mean ± SD of three experiments; B: Migration of HCC-LM3 cells on 200 nmol/L GST, FBLN-5 or FN. Monolayers were wounded and imaging at the leading edge was analyzed after 48 h of migration. Distances of wound width between 0 h (a) and 24 h (b) were counted and the mean ± SD is expressed as a percentage of cell migration by wound healing assays; C: The HCC-LM3 cell proliferation on GST (black dotted line), FBLN-5 (grey dashed line), FBLN-5 (black dashed line), and FN (black solid line) was investigated by CCK-8 assay. The y-axis represents the proliferation rate, calculated as the ratio to GST group (oh). Values are given as mean ± SD.


A
HCC-LM3
Relative expression levels (normalized to GAPDH)
10000
1000
100
10
0
pCDNA
FBLN-5-WT
FBLN-5-RGE
B
Hep3B
Relative expression levels (normalized to GAPDH)
1.2
1
0.8
0.6
0.4
0.2
0
NC
siRNA-576
siRNA-962
siRNA-1195
Cytoplasm
V5
β-actin
MMP-7
Supernatant
V5
Cytoplasm
Fibulin-5
β-actin
MMP-7
P = 0.2283
P = 0.0027
P = 0.0091
MMP-7 (ng/mL)
60
40
20
0
pCDNA
FBLN-5-WT
FBLN-5-RGE
P = 0.0009
P = 0.0097
P = 0.0047
MMP-7 (ng/mL)
50
40
30
20
NC
siRNA-576
siRNA-962
siRNA-1195
C
HCC-LM3 cells migration
pCDNA
FBLN-5-WT
FBLN-5-RGE
P = 0.0745
P = 0.0038
Migrating cells (normalized to pcDNA)
1.5
1.0
0.5
0.0
pCDNA
FBLN-5-WT
FBLN-5-RGE

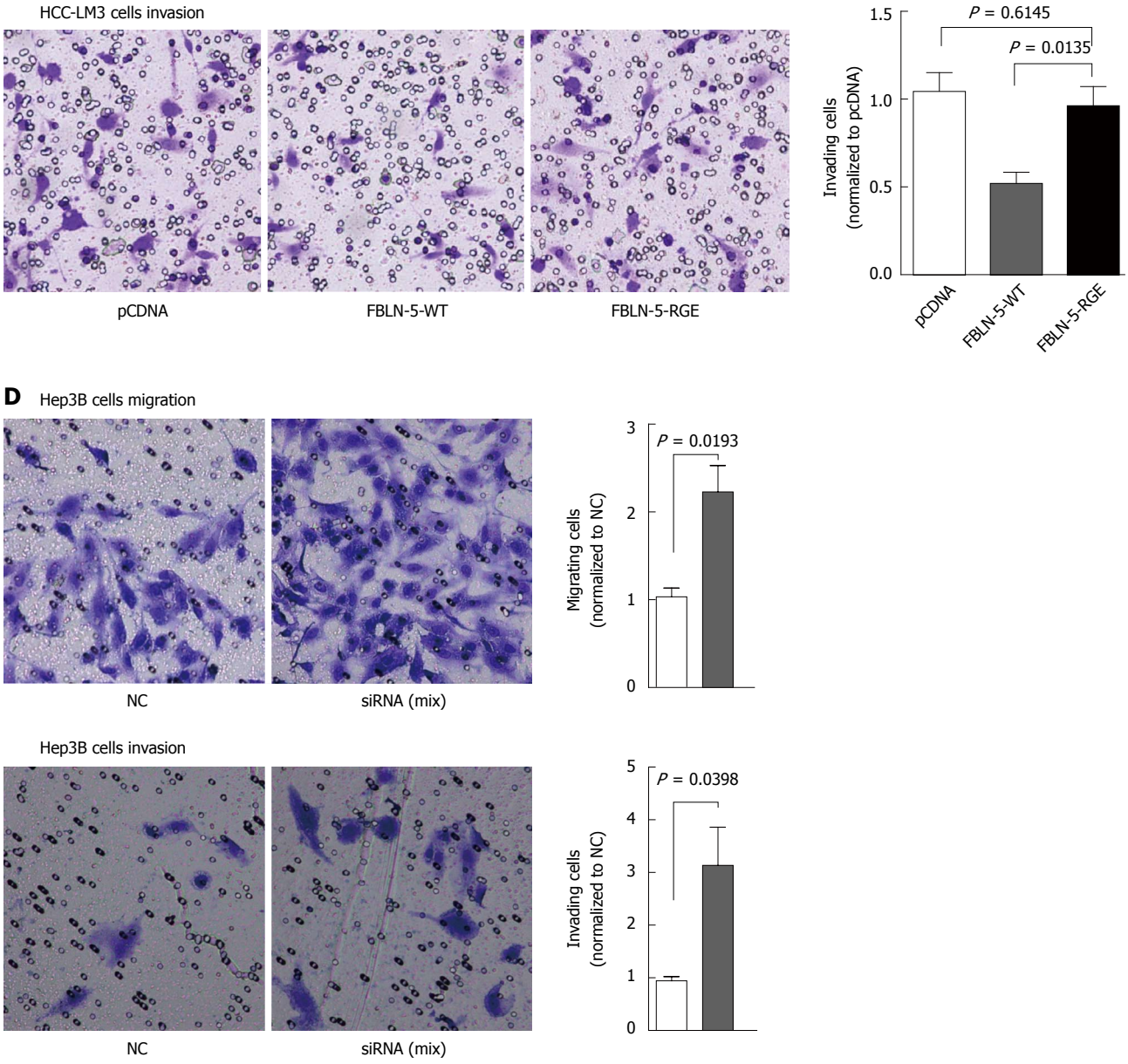


Figure 5 RGD-dependent suppression of hepatocellular carcinoma cell migration and invasion by fibulin-5 *via* matrix metalloproteinase 7. A: V5-tagged fibulin-5 (FBLN-5) was transfected into hepatocellular carcinoma (HCC)-LM3 cells, and V5-FBLN-5 expression was determined by Western blot with anti-V5 and anti-β-actin antibodies both in the cytoplasm and supernatant. The mRNA expression level of FBLN-5 was also measured by Q-RT-PCR. Matrix metalloproteinase 7 (MMP-7) expression was analyzed by Western blot 36 h after transfection. The levels of secreted MMP-7 were determined by ELISA (bottom) 36 h after transfection; B: A negative control siRNA (NC) plus FBLN-5 siRNA (siRNA-576, siRNA-962, siRNA-1195) was transfected into Hep3B cells for 48 h. After transfection, Western blot was performed with anti-FBLN-5 and β-actin antibodies. The mRNA expression level of endogenous FBLN-5 was also measured by Q-RT-PCR. MMP-7 expression was analyzed by Western blotting 36 h after transfection. The levels of secreted MMP-7 were determined by ELISA (bottom) 36 h after transfection; C: Migration and invasion of pcDNA-HCC-LM3, FBLN-5-WT-HCC-LM3 and FBLN-5-RGE-HCC-LM3 cells (× 100). For the migration assays, cells (pcDNA, FBLN-5-WT and FBLN-5-RGE) were seeded into the top of a transwell insert. After 24 h, the cells on top were scraped, and the cells that had migrated to the bottom were fixed and stained with crystal violet. The relative-fold migration values for the HCC-LM3 cells were normalized against the pcDNA and are represented diagrammatically. For the invasion assays, cells were seeded after the addition of matrigel. The relative-fold invasion values for the HCC-LM3 cells were normalized against the pcDNA and are represented diagrammatically; D: Migration and invasion results of NC and siRNA-FBLN-5 Hep3B cells are shown (× 100). The relative-fold migration and invasion values for siRNA-FBLN-5 transfected cells were normalized against the values for the NC and are represented diagrammatically. The results represent the mean ± SD of three independent experiments.

HCC-LM3 cells were plated at confluence on 200 nmol/L of GST, FBLN-5 or FN. The wound healing assays revealed that HCC-LM3 cells migrated to a lesser extent in the presence of FBLN-5 than FN ($P < 0.001$; Figure 4B). To investigate whether the adhesion of HCC-LM3 cells to FBLN-5 influences proliferation, the cells (2×10^3/well) were plated on 200 nmol/L FBLN-5 or FN and were counted on 2 and 4 d. Coincidentally, the proliferation of HCC-LM3 cells plated on FBLN-5 was significantly less than that of cells plated on FN (Figure 4C). These results show that in the presence of elevated FBLN-5/FN levels, FBLN-5 directly inhibits FN-

mediated migration and proliferation.

FBLN-5-mediated inhibition of HCC cell migration and invasion by downregulation of MMP-7 in an integrin-dependent manner

Based on the biochemical properties of FBLN-5 and the presence of the RGD motif[14], we hypothesized that it is involved in regulating cancer cell migration and invasion. The inverse correlation between FBLN-5 and MMP-7 expression has been reported previously in several types of cancer[5,15]. HCC-LM3 cells transfected with EV, FBLN-5-WT or FBLN-RGE were subjected to immunoblot analysis and ELISA to determine the levels of MMP-7 protein. FBLN-5-WT containing the RGD motif suppressed MMP-7 protein expression. In contrast, the FBLN-5-RGE mutant restored most of the MMP-7 expression and 40% of the level of secreted MMP-7 ($P < 0.05$; Figure 5A), which suggests that the inhibition of MMP-7 by FBLN-5 is mediated by the RGD motif through integrin signaling. Furthermore, knockdown of FBLN-5 resulted in an obvious increase in the level of MMP-7 protein in Hep3B cells ($P < 0.05$; Figure 5B).

We next evaluated the effect of FBLN-5 on HCC cell invasion and migration in transwells with or without matrigel. The representative fields showing cell migration and invasion are shown (Figure 5C and D). Overexpression of FBLN-5 in HCC-LM3 cells caused a significant decrease in cell migration and invasion ($P < 0.05$, respectively; Figure 5C), while knockdown of FBLN-5 resulted in an obvious increase in Hep3B cell migration and invasion ($P < 0.05$, respectively; Figure 5D). These findings suggest that the RGD motif of FBLN-5 plays an important role in human HCC cell migration and invasion *via* inhibition of MMP-7 expression.

DISCUSSION

Metastasis, which differs from tumor initiation, is the most lethal characteristic of HCC and is the main cause of HCC-related death. Matricellular proteins participate in matrix-cell interactions and exert regulatory roles *via* a variety of molecular mechanisms[16]. The importance of FBLN-5 in many human malignancies, such as nasopharyngeal carcinoma, lung and breast cancers, has been shown in mouse models and studies of various diseases[2], and recently, Tu *et al*[15] demonstrated that FBLN-5 inhibited the migration and invasion of HCC cells *via* downregulation of MMP-7 expression in 86 HCC tissues samples. However, as an extracellular matrix protein, precisely how FBLN-5 interacts with HCC cells and influences their functions remains unclear. Besides, more clinical tissue samples are required to confirm loss of FBLN-5 expression in HCC cells. In this study, we examined the interaction of FBLN-5 with HCC cells, and how these interactions regulate their behavior. We found that HCC cells adhere to FBLN-5 *via* integrin binding, and that their migration and invasion are inhibited by downregulation of MMP-7 *via* an integrin-binding RGD motif-dependent mechanism. We present the results of a systematic survey of FBLN-5 expression in 140 samples of HCC tissues, which is twice the number of samples studied in Tu's paper[15].

Our results indicate that the mRNA and protein levels of FBLN-5 are decreased in HCC tissues. However, the MSP assay showed that methylation of the FBLN-5 promoter was not found in any of the tumor samples or HCC cell lines, which indicates that the downregulation of FBLN-5 might not be related with any epigenetic alterations. Reduced expression of FBLN-5 was associated with clinically aggressive HCC and correlated with the number of tumor nodules, BCLC tumor stage, and TNM stage. Univariate analysis showed that the 5-year survival rate of HCC patients with low FBLN-5 expression in tumor cells was significantly lower than that in HCC patients with high FBLN-5 expression. These results suggest that reduced expression of FBLN-5 can be used as a predictor of poor prognosis of HCC and may play a role as a tumor suppressor in human HCC.

The ability of cancer cells to migrate and invade the basement membrane and the surrounding tissues, blood, and lymphatic vessels is an important characteristic of cancer and is a prerequisite for progression and metastasis of tumors[17]. Here, we found that exogenous FBLN-5 in HCC cells or HCC cells stimulated with the FBLN-5 protein could inhibit the proliferation, migration and invasion of the cells in culture systems. Conversely, after transfection with FBLN-5 siRNA, the number of cells migrating through or invading the filter significantly increased; this suggests that FBLN-5 downregulates human HCC cell proliferation, migration, and invasion. Compared with other studies[5,9,12,15,18-20], the current study indicates that FBLN-5 functions as a tumor suppressor or oncogene in various cancers in a context-specific manner.

Several studies have shown that the integrin family of transmembrane proteins is involved in the adhesion of cells with their extracellular matrix, which includes FN; moreover, integrins are known to play important roles in multiple features of cell physiology, including proliferation, migration and invasion[21,22]. In this study, we found that although FLBN-5 is ligated to the same integrins as FN, it exhibits considerably different cellular effects from FN and can modulate FN-mediated adhesion and migration. These results explain how FBLN-5 inhibits HCC migration and invasion as a component of the extracellular matrix. It has been reported that the effects of FBLN-5 on HCC cells are mediated by the inhibition of MMP-7, which is up-regulated and associated with poor prognosis of HCC[23,24]. In our study, we found that this molecular mechanism underlying the interaction between FBLN-5 and MMP-7 is mediated by integrin. The RGD motif of FBLN-5 binds to the integrins $\alpha v\beta 3$, $\alpha v\beta 5$, and $\alpha 9\beta 1$

in CHO cells[3], but the specific integrin subunit(s) that mediate the inhibitory effect of FBLN-5 on MMP-7 in HCC still need to be identified.

FBLN-5 has been identified as a novel target gene for TGF-β in fibroblasts[18], endothelial cells[18,25] and epithelial cells[9]. We found that TGF-β results in significant upregulation of FBLN-5 expression in HCC cells, while the tumor-promoting function of TGF-β is suppressed by FBLN-5 both in HCC-LM3 and Hep3B cells. Further investigation is required to examine the molecular connections potentially linking FBLN-5 to the acquisition of oncogenic signaling by TGF-β.

In summary, our findings suggest that down-regulation of FBLN-5 is a common abnormality in HCC and is correlated with HCC progression and poor survival. Recombinant full-length human FBLN-5 promoted the attachment of HCC cells *via* integrins, and inhibited HCC cell adhesion and migration to fibronectin in a concentration-dependent manner. Our results further indicate that FBLN-5 functions as a suppressor of HCC metastasis *via* an integrin-dependent mechanism by downregulating MMP-7. Although much work needs to be carried out to fully understand the function of FBLN-5 in the control of HCC progression, the validation of its relevance *in vitro* is an intriguing finding for cancer therapy.

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COMMENTS

Background

Fibulin-5 (FBLN-5) is characterized by an integrin-binding arginine-glycine-aspartic acid (RGD) motif that can bind certain integrins and regulate cell motility; it regulates the expression of several matrix metalloproteinases and tissue inhibitors of metalloproteinase in various cancers in a context-specific manner. However, the roles of FBLN-5 in hepatocellular carcinoma (HCC) remain to be fully elucidated.

Research frontiers

Previous experiments have revealed that the effects of FBLN-5 on HCC cells are mediated *via* the inhibition of matrix metalloproteinase 7 (MMP-7). Here, the authors showed that although FLBN-5 is ligated to the same integrins as FN, it has considerably different cellular effects and can modulate FN-mediated adhesion and migration. These results explain how FBLN-5 inhibits HCC migration and invasion as a component of the extracellular matrix.

Innovations and breakthroughs

This is the first study to demonstrate that recombinant full-length human FBLN-5 promotes the attachment of HCC cells *via* integrins. FBLN-5 inhibits HCC cell adhesion and migration to fibronectin in a concentration-dependent manner. In contrast, after transfection with FBLN-5 siRNA, the number of cells that migrated through or invaded the filter significantly increased; this suggests that FBLN-5 downregulates cancer cell proliferation, migration, and invasion in human HCC. These results provide further evidence for the role of FBLN-5 as a suppressor of HCC metastasis *via* an integrin-dependent mechanism involving downregulation of MMP-7.

Applications

This systematic survey of FBLN-5 expression in 140 samples of HCC tissues demonstrated that lower FBLN-5 expression is an important indicator of poor survival and that FBLN-5 inhibits HCC motility *via* an integrin-dependent mechanism; further, RGD-dependent suppression of MMP-7 by FBLN-5 might contribute to the development of new therapeutic strategies for HCC treatment.

Peer-review

The aim of this work was clear, and the authors performed plenty of experiments. It would be better to explain the reason for replacement of Asp56 within the integrin-binding RGD motif with Glu. It was assumed that this mutation weakened tumor suppression of FBNL-5. It would be better to introduce the relation between FBLN-5 and cell migration. Based on the results of this study, down-regulation of FBLN-5 seemed to promote cell motility and proliferation.

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ORIGINAL ARTICLE

Case Control Study

Low-density lipoprotein receptor genetic polymorphism in chronic hepatitis C virus Egyptian patients affects treatment response

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Data sharing statement: The technical appendix, statistical code, and dataset are available from the corresponding author at monasleman@kasralainy.edu.eg. No additional data are available.

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Abstract

AIM: To correlate a genetic polymorphism of the low-density lipoprotein (LDL) receptor with antiviral responses in Egyptian chronic hepatitis C virus (HCV) patients.

METHODS: Our study included 657 HCV-infected patients with genotype 4 who received interferon-based combination therapy. Patients were divided into two groups based on their response to therapy: 356 were responders, and 301 were non-responders. Patients were compared to 160 healthy controls. All patients and controls underwent a thorough physical examination, measurement of body mass index (BMI) and the following laboratory tests: serum alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, albumin, total bilirubin, direct bilirubin, prothrombin time, prothrombin concentration, INR, complete blood count, serum creatinine, fasting blood sugar, HCV antibody, and hepatitis B surface antigen. All HCV patients were further subjected to the following laboratory tests: HCV-RNA using quantitative polymerase chain reaction (PCR), antinuclear antibodies, thyroid-stimulating hormone, an LDL receptor (LDLR) genotype study of LDLR exon8c.1171G>A and exon-10c.1413G>A using real-time PCR-based assays, abdominal ultrasonography, ultrasonographic-guided liver biopsy, and histopathological examination of liver biopsies. Correlations of LDL receptor polymorphisms with HAI, METAVIR score, presence of steatosis, and BMI were performed in all cases.

RESULTS: There were no statistically significant differences in response rates between the different types of interferon used or LDLR exon10c.1413G>A. However, there was a significant difference in the frequency of the LDL receptor exon8c.1171G>A genotype between cases (AA: 25.9%, GA: 22.2%, GG: 51.9%) and controls (AA: 3.8%, GA: 53.1% and GG: 43.1%) ($P < 0.001$). There was a statistically significant difference in the frequency of the LDLR exon 8C:1171 G>A polymorphism between responders (AA: 3.6%, GA: 15.2%, GG: 81.2%) and non-responders (AA: 52.2%, GA: 30.6%, GG: 17.2%) ($P < 0.001$). The G allele of LDL receptor exon8c.1171G>A predominated in cases and controls over the A allele, and a statistically significant association with response to interferon was observed. The frequency of the LDLR exon8c.1171G>A allele in non-responders was: A: 67.4% and G: 32.6 *vs* A: 11.2% and G: 88.8% in responders ($P < 0.001$). Therefore, carriers of the A allele exhibited a 16.4 times greater risk for non-response. There was a significant association between LDL receptors exon8 c.1171G>A and HAI ($P < 0.011$). There was a significant association between LDL receptors exon8c.1171G>A and BMI. The mean BMI level was highest in patients carrying the AA genotype (28.7 ± 4.7 kg/m^2) followed by the GA genotype (28.1 ± 4.8 kg/m^2). The lowest BMI was the GG genotype (26.6 ± 4.3 kg/m^2) ($P < 0.001$). The only significant associations were found between LDL receptors exon8 c.1171G>A and METAVIR score or steatosis ($P < 0.001$).

CONCLUSION: LDL receptor gene polymorphisms play a role in the treatment response of HCV and the modulation of disease progression in Egyptians infected with chronic HCV.

Key words: Hepatitis C virus; Genetic polymorphisms; Low-density lipoprotein receptor; Egypt; Hepatitis C virus response to treatment

Core tip: Two molecules may function as hepatitis C virus (HCV) receptors, namely the low density lipoprotein receptor (LDLR) and CD81. This work assessed the role of genetic polymorphisms of the LDLR in Egyptian chronic HCV patients and its correlation with antiviral responses to treatment with pegylated interferon /ribavirin therapy. The study demonstrated that LDLR gene polymorphisms play a role in the response to viral treatment. The G allele of LDLRs exon8c.1171G>A predominated in cases and controls over the A allele. Carriers of the A allele exhibited a 16.4 times greater risk for non-response.

Naga M, Amin M, Algendy D, Elbadry A, Fawzi M, Foda A, Esmat S, Sabry D, Rashed L, Gabal S, Kamal M. Low-density lipoprotein receptor genetic polymorphism in chronic hepatitis C virus Egyptian patients affects treatment response. *World J Gastroenterol* 2015; 21(39): 11141-11151 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i39/11141.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i39.11141

INTRODUCTION

Hepatitis C exhibits a worldwide prevalence of approximately 3%[1]. Hepatitis C causes severe morbidity and mortality, and the majority of infected subjects fail to eliminate the virus and develop chronic hepatitis C. Chronic infections are estimated at approximately 80% of infected subjects, and this chronicity will lead to cirrhosis and /or hepatocellular carcinoma[2-8].

Egypt has the largest epidemic of hepatitis C virus (HCV) worldwide with a percentage of 14.7%, which is ten times greater than any other country[9-14].

The strong prevalence of HCV subtype (4a) in Egypt suggests that this chronic infection began as an epidemic. A history of injection treatment was implicated as a risk factor for HCV, and the prime culprit is the past practice of parental therapy for schistosomiasis[15,16].

The rate of disease progression varies widely, and it is not known what factors precisely determine the clinical outcome of the disease in the long term. The mechanism of viral entry into the host hepatocytes is not known.

Much effort was made to identify the receptors involved in viral entry into host cells.

Two molecules were proposed to function as HCV

receptors, namely the low density lipoprotein receptors (LDLRs) and CD81[17-20].

The LDLR gene family functions as a receptor for the minor group of common cold viruses and other viruses[21,22].

LDLR plays an important role in cholesterol homeostasis[23,24], and it is firmly established that mutations and polymorphisms in the LDLR gene are associated with familial hypercholesterolemia, obesity and atherosclerosis[25-31].

A direct interaction of HCV E2 envelope protein and LDL mediates the binding and internalisation of lipoviroparticles by LDLR in conjunction with CD81[32]. LDLR, CD81 and HCV E2 are co-localized on cells[33], and viral binding and endocytosis are inhibited by free LDL, free recombinant LDLR peptides, and antibodies against E2 and LDLRs[32,34]. Moreover, intracellular HCV RNA levels positively correlate with LDLR expression in cultured primary hepatocytes and *in vivo*[34,35]. These findings demonstrate that LDLRs are crucial co-receptors for HCV cell entry and replication. Furthermore, the role of LDLRs in immune responses and the incrimination of LDLR polymorphisms in disease processes makes these receptors an important candidate for the study of the genetic susceptibility to hepatitis C.

Several studies investigated nine single nucleotide polymorphisms in the *LDLR* gene and their possible influence on clinical parameters of HCV infection, such as viral clearance, overall inflammation, fibrosis severity and treatment response[36-38].

Our study assessed genetic polymorphisms of LDLR in chronic HCV genotype 4 Egyptian patients and the effects of polymorphisms on antiviral response.

MATERIALS AND METHODS

The review board of the Department of Internal Medicine, Faculty of Medicine, Cairo University approved the study protocol, which was performed according to the Declaration of Helsinki.

All study participants provided informed written consent prior to study enrolment. The study included 657 chronic HCV patients who were candidates for pegylated-interferon/ribavirin therapy and 160 healthy age- and sex-matched subjects as a control group. Patients were recruited from the outpatient clinics of the Internal Medicine Department, Kasr Al Aini Hospital, Cairo University, Beni-Suef Public Hospital and the National Liver Institute.

All patients and control group underwent thorough physical examinations, measurements of body mass index (BMI) and the following laboratory tests: serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), albumin, total bilirubin, direct bilirubin, prothrombin time (PT), prothrombin concentration (PC), INR, complete blood count (CBC), serum creatinine, fasting blood sugar (FBS), HCV antibody (anti-HCV), and hepatitis B surface antigen (HBsAg).

All HCV patients were further subjected to the following tests: HCV-RNA using quantitative polymerase chain reaction (PCR), antinuclear antibodies (ANA), thyroid-stimulating hormone (TSH), LDLR genotype study, abdominal ultrasonography and ultrasonographic-guided liver biopsy.

Patients received a course of antiviral therapy that consisted of pegylated interferon subcutaneously once weekly and oral ribavirin (10.6 mg/kg) daily. Patients with a reduction of more than 2 logs in PCR results after 12 wk of treatment were deemed responders, and treatment was continued for a total of 48 wk. Patients with PCR scores that were not at least 2 logs lower than baseline after 12 wk of therapy were deemed non-responders. Responders were re-tested 6 mo after the end of therapy using PCR to ensure a sustained virological response (SVR). We divided the patients into two groups based on treatment response: non-responders (n = 301) and responders (n = 356).

Types of interferon used

The following types of pegylated interferon were used based on availability: Peginterferon alfa-2a (PEGASYS® 180 mcg) was used in 287 cases, Peginterferon alfa-2a (Reiferon Retard® 160 mcg) was used in 59 cases, and Peginterferon alfa-2b (PEGINTRON® 1.5 mcg/kg per week) was used in 311 cases.

Blood sampling

Venous blood (10 mL) was collected from each patient in EDTA vacuum whole blood sample tubes. Samples were divided into 2 parts: 5 mL for HCV and biochemical parameters assessments and the other 5 mL for molecular LDLR gene polymorphism assessments.

Biochemical markers assessment

AST, ALT, T. bilirubin, D. bilirubin, albumin, ALP, FBS and creatinine were assessed using commercially available kits (Randox Laboratories Limited, Country Antrim, United Kingdom). AFP was evaluated using an ELISA kit (DRG International Inc., Springfield, New Jersey, United States). HbA 1c, PC, PT and INR were detected using kits (Stanbio Laboratory, Boerne, TX, United States). HB, TLC, ANC and platelets were detected using a cell counter (Sysmex XT-4000*i* Automated Hematology Analyzer Lincolnshire, IL, United States).

HCV RNA extraction and quantitative PCR detection

HCV RNA was extracted from 140 μL serum using the QIAamp Viral RNA Mini Kit (QIAgen, Hilden, Germany). Absolute quantitation of the concentration of HCV RNA was based on an external standard curve (HCV Standards IU/mL) in the presence of an internal positive control (IPC). IPC was added to a mixture of

Table 1 The primers and probes used for the low density lipoprotein receptor genes

LDLR primers	exon 8 (c.1171G > A)	F5'-CTACAAGTGCCAGTGTGAGGAA-3'
		R5'_CCCACCACTCTGCTTGTAAGGCGTGAGGCCGCC-3'
		Allele 1 specific probe VIC-ACACGAAGGCCTGC-NFQ
		Allele 2 specific probe 6-FAM-ACACGAAGACCT GC-NFQ
	exon 10 (c.1413G > A)	F5'- CGGCGTCTCTTCCTATGACA-3'
		R5'- GTCCAGTAGATGTTGCTGTGGAT-3'
		Allele 1 specific probe VIC-ATCAGCAGGGAC ATC-NFQ
		Allele 2 specific probe 6-FAM-TCAGCAGAGACA TC-NFQ

lysis buffer and sample material during RNA extraction of clinical blood samples. TaqMan assay was used in the AgPath-ID™ One-Step RT-PCR kit (Applied Biosystems, Foster City, CA, United States). The One-Step RT-PCR kit included an enzyme mixture, buffer and detection enhancer for one-step quantitative reverse transcription PCR (qRT-PCR). Amplification was performed in 25 μL of reaction mixture containing 2 × TaqMan Universal RT-PCR Master Mix, 20 μmol/L of each primer and probes for the sample and ICP and 8.5 μL of extracted RNA. All samples were performed in duplicate. Amplification began with an incubation at 50 ℃ for two min with uracil N'-glycosylase to inactivate possible contaminating amplicons, followed by 45 ℃ for 10 min for cDNA synthesis using reverse transcriptase, and 10 min at 95 ℃ to activate the AmpliTaq Gold polymerase and inactivate uracil N'-glycosylase. The PCR cycling program consisted of 45 cycles of 15 s at 95 ℃ and 45 s at 60 ℃ (universal conditions).

DNA extraction

Total DNA was isolated from whole blood mononuclear cells (MNCs) using an extraction kit (Qiagene, United States) according to manufacturer's instructions. DNA purity (*A*260/*A*280 ratio) and concentration were calculated using spectrophotometry (dual wavelength Beckman, Spectrophotometer, United States). The extracted and purified DNA samples were stored at -80 ℃ for further use.

LDLR genotyping

LDLR allelic discrimination variants were genotyped using real-time PCR and the QuantiTect® Probe PCR Kit (Qiagen, Hilden, Germany) and a Step-One instrument (Applied Biosystems, Foster City, United States). PCR products were generated under standard conditions in a 15 μL reaction containing 1 × reaction buffer, 1-2 mmol/L $MgCl_2$, 0.32 mmol/L dNTPs, 0.12 μmol/L of forward, reverse primer and probes, 1 unit Taq polymerase, 50 ng DNA and dH_2O. The PCR thermal cycling profile included an initial incubation at 50 ℃ for 2 min, 15 min at 95 ℃, and 40 amplification cycles (15 s at 95 ℃, 1 min at 60-64 ℃). Table 1 lists primers and probes. Finally, 1 μL of each LDR amplification product was run on an ABI prism 310 sequencer (Applied Biosystems, Warrington, United Kingdom), and sample genotypes were analysed using Genotyper software programmes (Applied Biosystems).

DNA Sequencing: PCR product samples were sequenced with a forward primer using a Big Dye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, United States) according to the manufacturer's instructions. Briefly, PCR sequencing cycling reactions were performed in a final total volume of 20 μL, which included 8 μL Big Dye terminator, 3.2 μL of 1 pmol diluted forward primer, 1 μL PCR product and 7.8 μL nuclease free water. The thermal profile conditions were 94 ℃ for 4 min, 95 ℃ for 15 s, 55 ℃ for 30 s and 60 ℃ for 4 min for 25 cycles. The sequencing cycling PCR products were purified using centri-sep nucleic acid gel-purified columns (Life Technology, Invitrogen). Ten microlitre of Hi Di formamide were added to 10 μL of purified PCR products. The mix was denatured in a PCR thermal cycler (Biometra, Germany) at 95 ℃ for 5 min. The reactions were performed in an automatic Sequencer ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, Calif.). The sequences obtained were analysed using the GenBank BLAST tool. The sequences were edited and aligned using the BioEdit Sequence Alignment. Table 1 shows the primers and probes for the *LDLR* genes.

Liver biopsy

Liver biopsies were performed using 18-gauge Tru-Cut Biopsy Needles under ultrasonographic guidance with a 3.5-MHz convex probe of a GE LOGIQ® P5 ultrasound machine after 6 h of fasting under complete aseptic conditions and the use of a local anaesthesia (5 mL of 2% lidocaine). Patients' vital signs of all patients were controlled for 2-4 h after the procedure to control probable complications. Biopsy specimens were placed in 10% formaldehyde and transferred to our pathology laboratory on the same day.

Histopathological examination of liver biopsies

All liver core biopsies were fixed for 24 h in a 10% neutral-buffered formalin solution, processed in ascending grades of ethyl alcohol (70%, 90%, and 100%) and xylene, and placed in paraffin blocks. Three paraffin wax sections were cut at 5 microns and stained using the following reagents: (1) hematoxylin and eosin for routine histopathological examination, diagnosis and necro-inflammatory scoring; (2)

Masson Trichrome to detect the extent of fibrosis. This stain imparts a blue colour to collagen against a red background of hepatocytes and other structures. It highlights the presence and distribution of reactive fibrosis as a result of liver injury. It is used for the staging of chronic liver diseases; and (3) prussian blue is a common and reliable stain for the detection of iron, which appears as blue granules in the cytoplasm.

Necro-inflammatory scoring and staging of the studied cases was performed using a modified HAI score and Metavir systems[39].

Statistical analysis

Data obtained from the study were coded and entered using SPSS (Statistical package for social science) software version 21. Parametric data are summarized using means and standard deviation, and non-parametric data are summarized as medians and percentiles for quantitative variables. Frequencies and percentages were used for qualitative variables. Comparisons between groups were performed using the χ^2 and Fischer's exact tests for qualitative variables, and Student's *t* test and the non-parametric Mann-Whitney test were used to compare two groups. ANOVA and a non-parametric test (Kruskal Wallis test) were used to compare multiple groups. The odds ratio (OR) and 95%CI were calculated to estimate the strength of associations between each genotype and alleles and patients and controls. *P* values were considered significant when $P < 0.05$.

RESULTS

Patient characteristics

A total of 657 chronic active hepatitis C patients were used in this study. Mean patient age was 42.5 ± 10.2 years. There were 399 males and 258 females. The control population included 160 volunteers. Patients were divided into 301 non-responders and 356 responders based on their responses to therapy. Median levels of BMI, ALT, and AST were significantly higher in the non-responders *vs* responders. There were no statistically significant differences in levels of albumin, viral load, platelet count, or ANA, and the presence of co-morbidities or the type of interferon used between groups. Liver biopsies from patients prior to treatment revealed a significant difference between responders and non-responders. Responders exhibited fewer disturbances in liver architecture as evidenced by HAI stage and Metavir A and F. There was also a significantly greater percentage of non-responders who exhibited liver steatosis than responders (Figures 1 and 2).

Frequency of LDLR genotype polymorphisms between responders, non-responders and controls

The frequency of the LDLR exon 8 C: 1171 G>A in the patient group was distributed as follows: AA: 170 (25.9%), GA: 146 (22.2%), and GG: 341 (51.9%). This result was significantly different from the control group: AA: 6 (3.8%), GA: 85 (53.1%) and GG: 69 (43.1%). Responders exhibited the following frequency of LDLR exon 8C:1171 G>A polymorphism: AA: 13 (3.6%), GA: 54 (15.2%), and GG: 289 (81.2%). Non-responders exhibited the following distribution: AA: 157 (52.2%), GA: 92 (30.6%), and GG: 52 (17.2%). These differences were statistically significant ($P < 0.001$).

LDLR exon 10 C1413 G>A revealed no significant differences between cases and controls, and there was no difference between responders and non-responders ($P = 0.354$) (Table 2).

Allele frequency and risk ratio in non-responders vs responders

There was a statistically significant association between the A allele of LDLR exon 8 C1171 G>A gene polymorphism and the response to treatment (standard of care treatment). Carriers of the A allele exhibited a 16.4 times greater risk for non-response to treatment than carriers of the G allele ($P < 0.001$). There was no significant association between response to therapy and LDLR exon 10 C:1413 G>A (Table 3).

Comparisons of HAI stage, BMI, steatosis and Metavir score to LDLR gene polymorphisms

The LDLR exon 8 C1171 G>A polymorphism was significantly associated with the HAI stage of liver fibrosis. There was a statistically significant association between the AA variant of the LDLR exon 8 genotype and increased BMI. There was no significant association between the different genotypes of LDLR exon 10 polymorphisms and liver fibrosis as evidenced by HAI and Metavir scores. There was a statistically significant association between LDLR exon 8 and Metavir A and F scores and the pathological evidence of steatosis. Carriers of the AA genotype exhibited a significantly higher incidence of fibrosis and steatosis. There was no association between the various genotypes of LDLR exon 10 gene polymorphisms and Metavir A or F scores or the presence of steatosis (Figure 3 and Table 4). There was no association between LDL receptor genotypes and the type of interferon in responders and non-responders.

DISCUSSION

Many studies suggested that HCV likely utilizes cell receptors to facilitate entry into cells[40]. The discrepancy in the rate of progression and clinical outcome of this disease was extensively investigated, and this step of viral entry is most incriminated in the development of chronicity[17]. The LDL receptor is a transmembrane glycoprotein that acts as a receptor for the uptake of cholesterols containing serum lipoproteins[41].

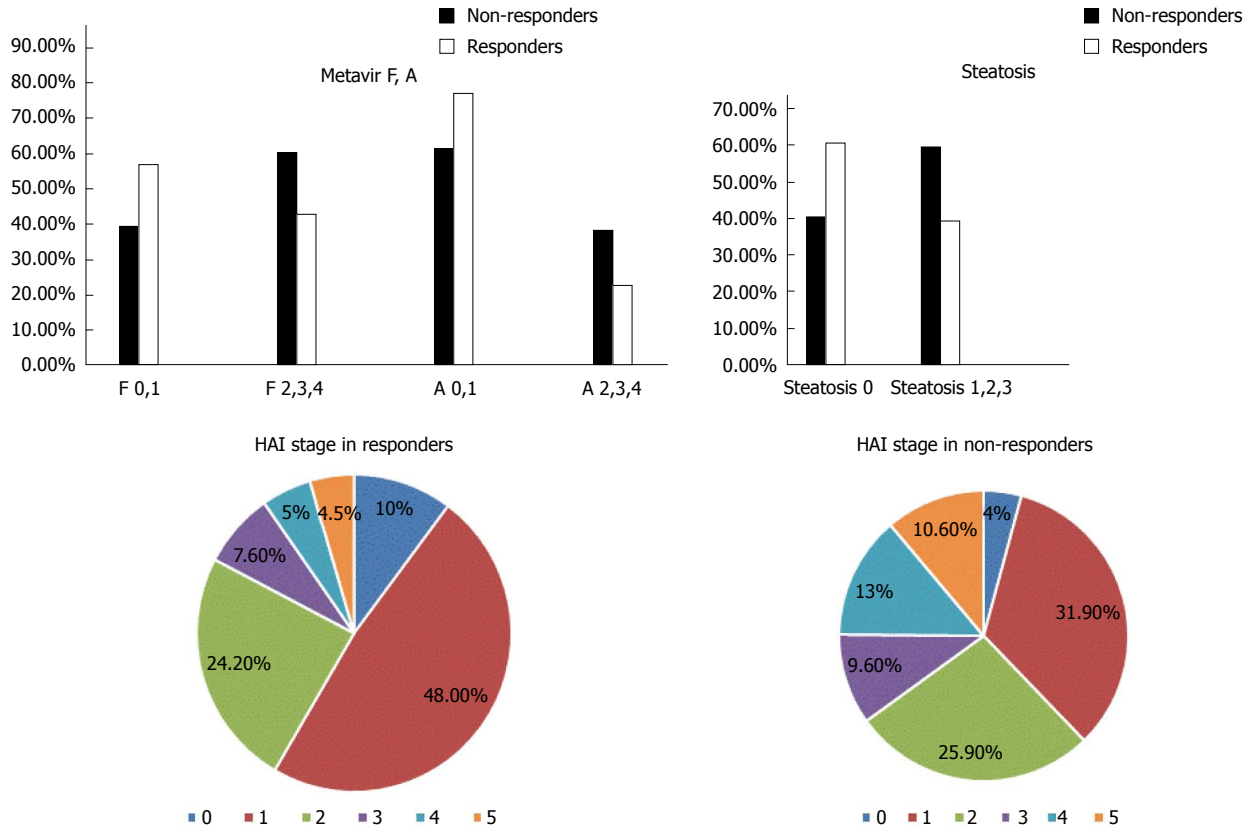

Figure 1 The HAI stage, Metavir score, steatosis grade in the responders and non-responders.

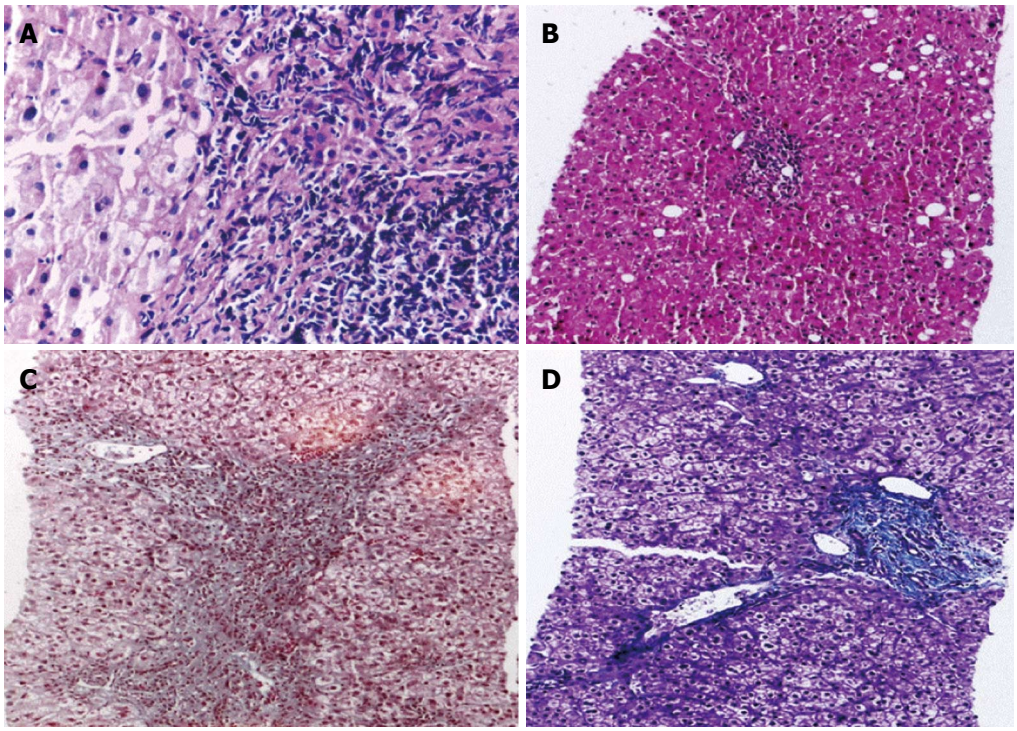

Figure 2 Histopathology of the liver biopsy tissues of patient group. A: Hematoxylin-eosin (HE) stain showing Portal tract s with piecemeal necrosis; B: HE stain showing moderate portal inflammation, the hepatocytes shows mild steatosis; C: MassomTrichrom stain showing Portal tract with bridging fibrosis; D: MassomTrichrom stain showing fibrous expansion with septation.

Table 2 The frequency of the genotypes of gene polymorphisms in the studied groups *n* (%)

	Non responders (*n* = 301)	Responders (*n* = 356)	Control (*n* = 160)	*P* value
LDR exon8 c.1171G>A				< 0.001
AA	157 (52.2)	13 (3.6)	6 (3.8)	
GA	92 (30.6)	54 (15.2)	85 (53.1)	
GG	52 (17.2)	289 (81.2)	69 (43.1)	
LDR exon10 c.1413G>A				0.204
AA	48 (15.9)	51 (14.3)	15 (9.4)	
GA	191 (63.5)	215 (60.4)	109 (68.1)	
GG	62 (20.6)	90 (25.3)	36 (22.5)	

Table 3 Allele frequency and risk ratio in non-responders *vs* responders

	Non responders *n* (%)	Responders *n* (%)	*P* value	OR (95%CI)
LDR exon 8 c.1171G>Aallele				
A	406 (67.4)	80 (11.2)	< 0.001	16.4 (12.3-21.8)
G	196 (32.6)	632 (88.8)		1
exon 10 c.1413 G>Aallele				
A	287 (47.7)	317 (44.5)	0.253	1.13 (0.9-1.4)
G	315 (52.3)	395 (55.5)		1

The LDL receptor is a proposed receptor for HCV entry into hepatocytes. However the exact mechanism of HCV entry is not clear. The association of the virus with a host's lipoprotein component may facilitate this interaction[42]. The endoplasmic reticulum is the likely site of production of lipid droplets that associate with HCV after its replication and assembly[43]. Mature viruses are released from infected cells, and they are found in the low-density fraction of the serum of infected persons[44]. Hepatocytes endocytosed HCV-RNA in the LDL fraction of HCV-infected patients. Anti-LDL and anti-ApoE antibodies block viral entry into cells[17]. Several in vitro studies confirmed this hypothesis and concluded that the binding and entry of HCV particles into cultured hepatocytes was strongly associated with the level of LDLR expression[45]. HCV infection propagates LDLR expression, which further promotes lipid uptake from the blood and enriches the hepatocyte content of lipids to facilitate HCV replication[44,46].

Some studies indicate that the LDLR functions as a receptor of HCV binding and entry through interactions between the HCV/lipoprotein virion complex[47]. Other studies suggest that the LDLR is essential only for post-entry events, such as viral replication[48]. These studies note the possible role of other host receptors in HCV binding, attachment, endophagocytosis and viral replication, whether sequentially or individually. However, the viral envelope glycoproteins E1 and E2 are the main binding sites for the host receptors[49].

Direct interaction of HCV_{E2} envelope proteins and LDL mediates the binding and internalisation of lipoviroparticles by LDLR[32]. Atherosclerosis, obesity and familial hypercholesterolaemia are definitely related to mutations and polymorphisms in the gene encoding for LDLR[25]. This fact led investigators to probe the significance of gene polymorphisms of LDLR and the development of fibrosis, and the response to the standard of care treatment.

This study assessed gene polymorphisms at exon 8 G>A 1171 and exon 10 G>A 1413. The study was conducted on 657 patients with HCV infection and 160 healthy controls. The genotype distribution and allele frequency of exons 8 and 10 of the LDLR was studied and related to liver inflammation, the presence of steatosis, and BMI.

Our results demonstrated that the AA gene polymorphism of the 3 types of gene polymorphisms found in exon 8 of the LDLR (AA, GA, GG) was significantly different ($P < 0.001$) between HCV patients and the healthy control group, but the GA and GG gene polymorphisms were not significantly different. LDLR exon 10 also revealed no significance in gene polymorphisms (either AA, GA or GG), which indicates that the presence of the AA gene polymorphism of LDLR exon 8 was clearly associated with a higher susceptibility to HCV infection. Furthermore, the LDLR exon 8 AA gene polymorphism occurred more frequently in non-responders of interferon-based treatment than responders, and this difference was highly statistically significant ($P < 0.001$). A total of 157 patients (52.2%) of the non-responders had AA genes, but only 13 (3.6%) of the responders had AA genes.

However, no such discrepancy was detected in patients with GA or GG genes. exon 10 also exhibited no differences between gene polymorphisms between responders and non-responders.

One of the most important outcomes of the present study was the statistically significant association between the A allele of LDLR exon 8 gene polymorphism and the response to interferon, with carriers of the A allele exhibiting a 16.4 times greater risk for non-response to interferon-based treatment than patients who did not carry the A allele ($P < 0.001$), regardless of the type of interferon used.

The study identified a significant association between LDLR exon 8 and HAI staging and Metavir A and Metavir F, but this statistically significant difference was not present in LDLR exon 10.

Steatosis and BMI levels were significantly associated with LDLR exon 8, and both factors were higher in patients carrying the AA genotype. However, no such association was found in LDLR exon 10.

This difference may be explained by the fact that polymorphisms in the LDLR gene theoretically affect steatosis, which eventually leads to more severe liver disease and the failure of response to treatment in patients with HCV. However, the relationship between

Table 4 Steatosis and body mass index mean level in different *LDLR* genotypes in hepatitis C virus patients *n* (%)

	LDR exon 8 c.1171 G>A			*P* value	LDR exon 10 c.1413 G>A			*P* value
	AA	GA	GG		AA	GA	GG	
BMI (kg/m^2)	28.7 ± 4.7	28.1 ± 4.8	26.6 ± 4.3	< 0.001	27.3 ± 4.3	27.5 ± 4.8	27.4 ± 4.4	0.887
Steatosis								
0	64 (37.6)	70 (47.9)	204 (59.8)	< 0.001	50 (50.5)	208 (51.2)	80 (52.6)	0.938
1,2,3	106 (62.4)	76 (52.1)	137 (40.2)		49 (49.5)	198 (48.8)	72 (47.4)	

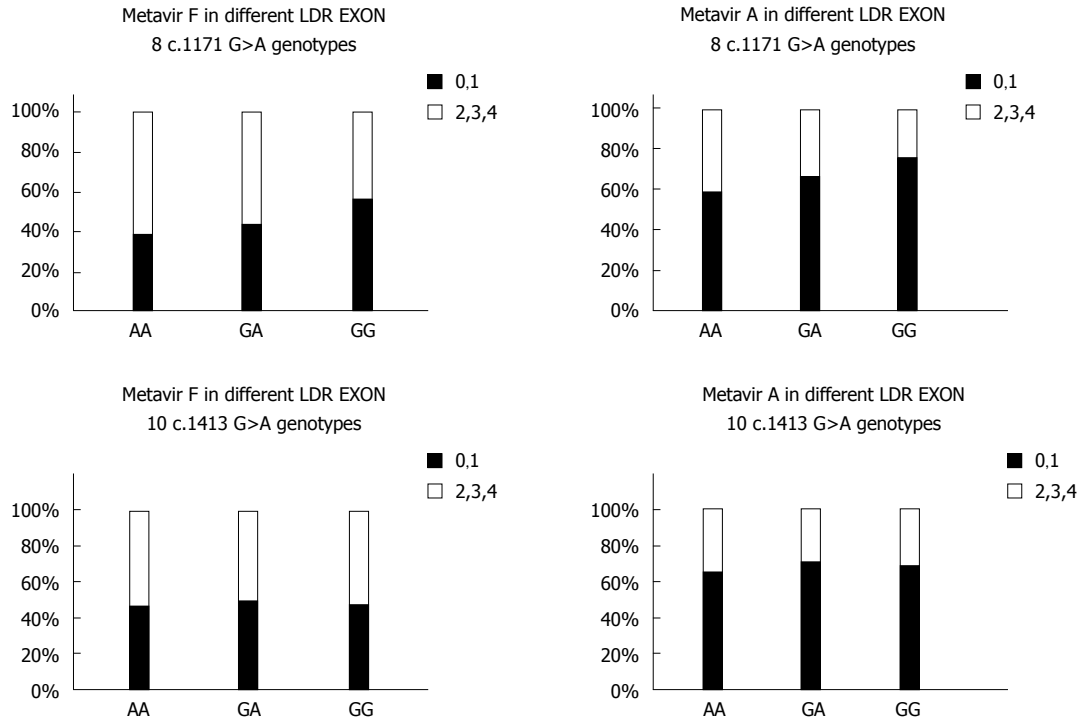

Figure 3 Low density lipoprotein receptor genes studied and Metavir A and F score in patient groups.

exon 8 and steatosis requires further study.

The finding that a single polymorphism in exon 8 was associated with more severe liver disease was also concluded in a study by Li *et al*[50] in 2006, who reported a correlation between exon 8 and fibrosis severity. In contrast to our study, Li *et al*[50] correlated a polymorphism of exon 10 to viral clearance and overall inflammation, which was not observed in our study. This difference may be explained by the difference in the ethnicity of the group studied and/or the type of viral genome.

The same correlation between exon 10, viral clearance and overall inflammation was also observed in a study by Hennig *et al*[36]. This study agreed with our study that the polymorphism in exon 8 exhibited the strongest association with disease severity. However the carriage of the G allele on exon 8 was associated with more severe fibrosis in this study, and heterozygosity at this site appeared protective against inflammation. This result directly contradicts our study in which the presence of the A allele was associated with more severe disease, and the presence of the G allele was somewhat protective.

A more recent study by Mas Marques *et al*[51] in 2009 demonstrated that the prevalence of an LDLR exon 8 polymorphism did not alter the grade of inflammation or stage of fibrosis. However, a careful analysis of this study reveals a low prevalence of the A allele of exon 8, which may support our results.

In conclusion, polymorphisms in the LDLR play a key role in the progression of HCV infection. The recent study of different exons identified exons 8 and 10 for further scrutiny. The results that relate polymorphisms of the alleles of these exons to the propagation of HCV infection and disease progression are somewhat contradictory. The need to clarify this dilemma is evident to improve disease outcome and treatment response in patients with HCV. This results of the present and previous studies supports the pivotal role of LDLR exon 8 in the natural course of HCV and its response to treatment. However, it is evident that further studies are needed to clarify whether other exons and alleles play a similar role.

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COMMENTS

Background

Hepatitis C virus (HCV) infection causes severe morbidity and mortality worldwide. Egypt has the largest epidemic of HCV worldwide. It was proposed that low-density lipoprotein receptors (LDLR) function as HCV receptors, and these receptors are implicated in the entry of HCV into hepatocytes.

Research frontiers

Genetic polymorphisms of the LDLR may be involved in treatment response and final disease outcome. Assessment of the frequency of LDLR genotype polymorphisms and the effect of these genetic polymorphisms in Egyptian chronic HCV patients aid predictions of treatment response.

Innovations and breakthroughs

The results demonstrated that the G allele of LDL receptor exon8c.1171G>A predominated in cases and controls over the A allele. Moreover, carriers of the A allele have a 16.4 times greater risk for non-response to anti-HCV therapy. A significant association was also found between LDL receptor exon8 c.1171G>A and the degree of liver fibrosis.

Applications

These findings aid in the selection of patients who will benefit from anti-HCV therapy. The study also confirmed the important role of LDLR exon 8 in the natural course of HCV and patient response to treatment. However, further studies are needed to clarify whether other exons and alleles play a similar role.

Terminology

LDLR is a cell surface receptor that plays an important role in cholesterol homeostasis. Mutations and polymorphisms in the LDLR gene are associated with familial hypercholesterolemia, obesity and atherosclerosis. The LDLR acts as a viral receptor. The LDLR gene family functions as a receptor for the minor group of common cold viruses, and these receptors may function as HCV and other virus receptors.

Peer-review

This report was very interesting study about HCV and LDLR polymorphism. HCV particles are known to be in complex with lipoproteins. Discussion need to be more detailed about LDLR and HCV.

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ORIGINAL ARTICLE

Case Control Study

Serum vitamin D_3 does not correlate with liver fibrosis in chronic hepatitis C

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Abstract

AIM: To investigate the relationship between serum vitamin D_3 levels and liver fibrosis or inflammation in treatment-naive Chinese patients with chronic hepatitis C (CHC).

METHODS: From July 2010 to June 2011, we enrolled 122 CHC patients and 11 healthy controls from Dingxi

city, Gansu Province, China. The patients were infected with Hepatitis C virus (HCV) during blood cell re-transfusion following plasma donation in 1992-1995, and had never received antiviral treatment. At present, all the patients except two underwent liver biopsy with ultrasound guidance. The Scheuer Scoring System was used to evaluate hepatic inflammation and the Metavir Scoring System was used to evaluate hepatic fibrosis. Twelve-hour overnight fasting blood samples were collected in the morning of the day of biopsy. Serum levels of alanine aminotransferase, aspartate aminotransferase, total bilirubin, direct bilirubin, cholinesterase, prothrombin activity, albumin, γ-glutamyl transpeptidase, hemoglobin, calcium and phosphorus were determined. Serum HCV RNA levels were measured by real-time PCR. Serum levels of 25-hydroxyvitamin D_3 [25(OH)D_3] and 24,25-dihydroxyvitamin D_3 [24,25(OH)$_2D_3$] were measured by high-performance liquid chromatography tandem mass spectrometry.

RESULTS: Serum levels of 25(OH)D_3 but not 24,25(OH)$_2D_3$ were significantly lower in CHC patients than in control subjects. Serum 25(OH)D_3 levels did not correlate with liver fibrosis, inflammation, patient age, or levels of alanine aminotransferase, aspartate aminotransferase, total bilirubin, direct bilirubin, prothrombin activity, cholinesterase or HCV RNA. However, serum 25(OH)D_3 levels did correlate with serum 24,25(OH)$_2D_3$ levels. Serum 25(OH)D_3 and 24,25(OH)$_2D_3$ levels, and the 25(OH)D_3/24,25(OH)$_2D_3$ ratio, have no difference among the fibrosis stages or inflammation grades.

CONCLUSION: We found that serum levels of 25(OH)D_3 and its degradation metabolite 24,25(OH)$_2D_3$ did not correlate with liver fibrosis in treatment-naive Chinese patient with CHC.

Key words: Hepatitis C virus; 25-Hydroxyvitamin D_3; 24,25-Dihydroxyvitamin D_3; Fibrosis; Inflammation

Core tip: We studied the relationship between liver fibrosis, based on liver biopsies, and serum vitamin D_3 levels in Chinese treatment-naive patients with chronic hepatitis C (CHC). The levels of serum 25-hydroxyvitamin D_3 [25(OH)D_3] were significantly lower in the patients than in healthy control subjects. The levels of the degradation metabolite 24,25-dihydroxyvitamin D_3 [24,25(OH)$_2D_3$] did not differ between the patients and controls. Spearman's rank correlation analysis indicated that serum 25(OH)D_3 levels did not correlate with the extent of liver fibrosis or inflammation. However, serum 25(OH)D_3 levels did correlate with serum 24,25(OH)$_2D_3$ levels. Serum 25(OH)D_3 and 24,25(OH)$_2D_3$ levels, and the 25(OH)D_3/24,25(OH)$_2D_3$ ratio, have no difference among the fibrosis stages or inflammation grades. In conclusion, the levels of serum 25(OH)D_3 and its metabolite 24,25(OH)$_2D_3$ did not correlate with liver fibrosis in treatment-naive Chinese patient with CHC.

Ren Y, Liu M, Zhao J, Ren F, Chen Y, Li JF, Zhang JY, Qu F, Zhang JL, Duan ZP, Zheng SJ. Serum vitamin D_3 does not correlate with liver fibrosis in chronic hepatitis C. *World J Gastroenterol* 2015; 21(39): 11152-11159 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i39/11152.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i39.11152

INTRODUCTION

Vitamin D has many functions in addition to regulating the metabolism of calcium and phosphorus, such as immunomodulatory and anti-proliferative activities[1]. The liver plays an important role in vitamin D metabolism. Vitamin D_3 is obtained through food intake, and 7-dehydrocholesterol in skin can be converted into vitamin D_3 after exposure to ultraviolet light. Vitamin D_3 is hydroxylated to 25-hydroxyvitamin D_3 [25(OH)D_3] in the liver. 25(OH)D_3 is further hydroxylated to 1,25-dihydroxyvitamin D3 [1,25(OH)$_2D_3$] by 1-α-hydroxylase in the kidneys. It is this form of vitamin D that binds to receptors and plays a biological role. Both 25(OH)D_3 and 1,25(OH)$_2D_3$ can be converted into 24,25-dihydroxyvitamin D_3 [24,25(OH)$_2D_3$], which is an inactive form of vitamin D_3[2].

Recent studies have found that there is a close relationship between vitamin D and hepatitis C[3-6]. Although it is accepted that patients with chronic hepatitis C (CHC) have low serum levels of 25(OH)D_3, there are inconsistencies regarding its role in liver fibrosis. Several studies have shown that the low serum levels of 25(OH)D_3 in CHC patients are associated with the progression or degree of liver fibrosis and the response of hepatitis C virus (HCV) to antiviral therapy[7,8]. In contrast, other studies have found no correlation between vitamin D levels and either liver fibrosis or sustained virological responses[9,10].

The association of 24,25(OH)$_2D_3$ levels with liver fibrosis in CHC has not been reported. This is an interesting issue that should be addressed, as 24,25(OH)$_2D_3$ is downstream in the vitamin D metabolic pathway.

Because vitamin D levels and fibrosis are affected by many factors such as race, diet, light exposure, measurement methods, and interferon treatment, there is an urgent need to evaluate the role of vitamin D in liver fibrosis in patients with CHC. To date, the correlation between serum 25(OH)D_3 levels and liver fibrosis, based on liver biopsy, has rarely been reported in Chinese Han patients with CHC.

In this study, we enrolled 122 patients with CHC who were naïve to antiviral treatment and 11 healthy

controls. All of the subjects were of Han ethnicity and living in the same area of Dingxi, Gansu Province, with similar environmental and eating habits. The purpose of this study was to measure 25(OH)D_3 and 24,25(OH)$_2D_3$ serum levels in Chinese Han patients with CHC, and to analyze the correlation between these levels and liver fibrosis or inflammation.

MATERIALS AND METHODS

Patients

From July 2010 to June 2011, we enrolled 122 CHC patients and 11 healthy controls from Dingxi city, Gansu Province, China. These patients were infected with HCV during blood cell re-transfusion following plasma donation in 1992-1995, and had never received antiviral therapy. The diagnosis of CHC was made according to the established criteria[11,12]. Patients were excluded from the study if they had liver disease of mixed or other etiology, such as hepatitis B, autoimmune liver disease, excessive alcohol consumption, or Wilson's disease, or if the patients had hepatocellular carcinoma or other malignant diseases. Patients with vitamin D supplementation or other medical therapy that influences vitamin D metabolism were also excluded.

All CHC patients and healthy controls gave informed consent before the study. The study protocol was in accordance with the provisions of the Declaration of Helsinki and its appendices. This study was approved by the Institutional Review Board of Beijing YouAn Hospital, Capital Medical University, Beijing, China.

Liver histology

CHC patients underwent liver biopsy with ultrasound guidance. The biopsy specimens had a minimum length of 15 mm and contained at least six complete portal tracts. Two patients were excluded from pathological analysis: one because of ascites, and the other because the liver biopsy was too small. Thus, 120 patients were included for further assessment. The liver sections stained by hematoxylin and eosin. Slides were coded and read by two pathologists who were unaware of the patient's identity and history. The Scheuer Scoring System was used to evaluate hepatic inflammation and the Metavir Scoring System was used to evaluate hepatic fibrosis. A fibrosis score of F $\geqslant$ 2 was defined as moderate fibrosis, and F $\geqslant$ 3 was defined as severe fibrosis.

Clinical and laboratory assessment

Twelve-hour overnight fasting blood samples were collected in the morning of the day of biopsy. Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), direct bilirubin (DBIL), cholinesterase, prothrombin activity (PTA), albumin, γ-glutamyl transpeptidase (GGT), calcium and phosphorus were determined by automated biochemical detection. Blood cell counts were determined by multi-parameter auto-calculator. Serum HCV RNA levels were determined by real-time PCR.

Serum 25(OH)D_3 and 24,25(OH)$_2D_3$ measurements

Blood samples for vitamin D measurements were collected at the same time as the blood samples for clinical parameter measurements. Serum samples were stored at -80 ℃, then 25(OH)D_3 and 24,25(OH)$_2D_3$ levels were measured by high-performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) at the State Key Laboratory of Bioactive Substance and Function of Natural Medicines, Institute of Materia Medica, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China. All vitamin D standards were purchased from Sigma (St Louis, MO, United States). HPLC-MS/MS was performed on an Agilent 6410B Triple Quad mass spectrometer (QQQ; Agilent Technologies, Santa Clara, CA, United States) containing a triple quadrupole MS analyzer with an electrospray ionization interface and an Agilent 1200 RRLC system.

Statistical analysis

Continuous variables were summarized as mean ± standard deviation and categorical variables were summarized as frequency and percentage. Mann-Whitney and Kruskal-Wallis tests were used to compare continuous variables when appropriate. Correlations were evaluated with the Spearman rank coefficient. All statistical tests were two-tailed, and $P < 0.05$ was considered statistically significant. We used SPSS version 18.0 (Chicago, IL, United States).

RESULTS

Clinical characteristics

The clinical features of the 120 patients who underwent liver biopsy and 11 healthy controls are shown in Table 1. The male-to-female ratio of the patients was 57/63, with a mean age of 51.33 ± 7.33 years. The Metavir fibrosis scores were: F0 (1/120, 0.8%), F1 (55/120, 45.8%), F2 (50/120, 47.1%), F3 (12/120, 10%), and F4 (2/120, 1.7%). The inflammation grades were: G0 (1/120, 0.8%), G1 (16/120, 13.3%), G2 (67/120, 55.8%), G3 (34/120, 28.3%), and G4 (2/120, 1.7%).

Serum 25(OH)D_3 and 24,25(OH) $_2D_3$ levels

The average level of serum 25(OH)D_3 in the patients with CHC was 5.84 ± 2.63 ng/mL, which was lower than that of the healthy controls (10.08 ± 3.15 ng/mL) ($z = -3.93$, $P < 0.001$). The average level of serum 24,25(OH)$_2$ D_3 did not differ significantly between the patients and controls (1.78 ± 1.02 ng/mL *vs* 2.30 ± 1.27 ng/mL, $z = -1.40$, $P = 0.161$). The ratio of 25(OH)D_3 to 24,25(OH)$_2D_3$ also did not differ significantly between the two groups (4.10 ± 2.44 *vs*

Table 1 Characteristics of patients and controls

Features	Patients (n = 120)	Controls (n = 11)
Male/female	57/63	4/7
Age (yr)	51.33 ± 7.33	38.45 ± 6.95
BMI (kg/m^2)	22.34 ± 2.73	24.00 ± 2.53
ALT (U/L)	60.42 ± 70.88	16.71 ± 5.14
AST (U/L)	47.94 ± 44.30	19.87 ± 3.49
TBIL (μmol/L)	16.51 ± 7.25	16.30 ± 5.64
DBIL (μmol/L)	3.26 ± 1.36	2.82 ± 0.75
TP (g/L)	70.93 ± 4.95	69.05 ± 3.15
ALB (g/L)	43.24 ± 2.36	43.86 ± 2.18
GGT (U/L)	22.04 ± 16.50	15.90 ± 10.82
CHE (U/L)	6657.09 ± 1442.44	7703.45 ± 945.75
Ca (mmol/L)	2.26 ± 0.08	2.21 ± 0.05
P (mmol/L)	0.92 ± 0.19	1.02 ± 0.14
HB (g/L)	151.09 ± 16.56	144.45 ± 11.43
PLT (× 10^9/L)	171.36 ± 53.20	242.18 ± 48.36
PTA (%)	92.41 ± 9.11	93.78 ± 4.69
Virus load, n (%)		
< 4 log10	20 (16.7)	
4-5 log10	52 (43.3)	
6-7 log10	48 (40.0)	
Stage of fibrosis, n (%)		
0	1 (0.8)	
1	55 (45.8)	
2	50 (47.1)	
3	12 (10)	
4	2 (1.7)	
Grade of inflammation, n (%)		
0	1 (0.8)	
1	16 (13.3)	
2	67 (55.8)	
3	34 (28.3)	
4	2 (1.7)	

BMI: Body Mass Index; ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; TBIL: Total Bilirubin; DBIL: Direct Bilirubin; TP: Total Protein; ALB: Albumin; GGT: Gamma-glutamyl Transpeptidase; CHE: Cholinesterase; HB: Hemoglobin; PLT: Platelets; PTA: Prothrombin Time Activity.

5.52 ± 2.47, P = 0.076) (Table 2).

Correlations between serum vitamin D_3 levels and clinical parameters in CHC patients

Spearman's rank correlation analysis showed that neither 25(OH)D_3 nor 24,25(OH)$_2D_3$ correlated with patient age or level of ALT, AST, TBIL, DBIL, PTA, cholinesterase, or HCV RNA. The 25(OH)D_3/24,25(OH)$_2D_3$ ratio correlated negatively with the serum levels of ALT (r = -0.230, P = 0.012), TBIL (r = -0.176, P = 0.054), DBIL (r = -0.282, P = 0.002), total protein (r = -0.185, P = 0.043), and GGT (r = -0.211, P = 0.021). Serum 25(OH)D_3 levels correlated positively with the levels of 24,25(OH)$_2D_3$ (r = 0.410, P < 0.001) (Table 3).

Serum vitamin D_3 levels and liver fibrosis/inflammation in CHC patients

Spearman's rank coefficient analysis showed that serum 25(OH)D_3 levels did not correlate with liver fibrosis (P = 0.574). Although serum 25(OH)D_3 levels exhibited some variation with fibrosis stage (F0-F1 *vs* F2 *vs* F3-F4, P = 0.027) (Figure 1A), the changing trend in serum 25(OH)D_3 levels was not consistent: 25(OH)D_3 levels were lower in F2 (5.12 ± 2.15 ng/mL) than in F0-F1 (6.17 ± 2.76 ng/mL) and F3-F4 (7.06 ± 3.10 ng/mL). Further analysis indicated that there was no difference in serum 25(OH)D_3 levels between non-severe and severe fibrosis patients (F0-F2 *vs* F3-F4) (Figure 1B) and between mild and apparent fibrosis patients (F0-F1 *vs* F2-F4) (Figure 1C).

Table 2 Serum 25(OH)D_3 and 24,25(OH)$_2D_3$ levels, and the 25(OH)D_3/24,25(OH)$_2D_3$ ratio, in chronic hepatitis C patients and healthy controls

	Patients (n = 120)	Controls (n = 11)	P value
25(OH)D_3 (ng/mL)	5.84 ± 2.63	10.08 ± 3.15	< 0.001
24,25(OH)$_2D_3$ (ng/mL)	1.78 ± 1.02	2.30 ± 1.27	0.161
25(OH)D_3/24,25(OH)$_2D_3$	4.10 ± 2.44	5.52 ± 2.47	0.076

To explore the association of serum 25(OH)D_3 levels with liver inflammation, we made multiple comparisons among the different liver inflammation subgroups. However, serum 25(OH)D_3 levels did not vary significantly among the inflammation grades (G0-G1 *vs* G2 *vs* G3-G4; or G0-G2 *vs* G3-G4; or G0-G1 *vs* G2-G4) (Figure 2).

Similarly, serum 24,25(OH)$_2D_3$ levels and the ratio of 25(OH)D_3 to 24,25(OH)$_2D_3$ have no difference among the fibrosis stages or inflammation grades (Figures 1 and 2).

DISCUSSION

We enrolled Chinese Han subjects from the same geographical area, with similar lifestyles, eating habits and living environments. All the CHC patients had the same route of infection, by plasma donation in 1992-1995, and had never received antiviral treatment. All factors influencing the level of vitamin D and liver fibrosis were adjusted to a similar level or eliminated as far as possible; therefore, we were able to obtain the true results of changes in serum vitamin D levels and their association with liver fibrosis. Liver biopsy and HPLC-MS/MS showed that the serum 25(OH)D_3 level had no correlation with fibrosis stage or inflammation grade in the Chinese Han CHC patients. To the best of our knowledge, this is the first study to show that there is no difference in serum 24,25(OH)$_2D_3$ levels between healthy individuals and CHC patients, and that the serum 24,25(OH)$_2D_3$ level does not correlate with liver fibrosis and inflammation.

Although increasing evidence has confirmed that CHC patients are deficient in vitamin D[13-15], the relationship between vitamin D deficiency and liver fibrosis is still unclear. There are several inconsistencies in the literature regarding the role of vitamin D in liver fibrosis[16]. Some studies found a correlation between vitamin D levels and hepatitis C progression[17-19], but other studies found the contrary[9,20,21]. The main

Table 3 Correlations of serum 25(OH)D_3 and 24,25$(OH)_2D_3$ levels, and the 25(OH)D_3/24, 25$(OH)_2D_3$ ratio, with clinical parameters

	25(OH)D_3 (ng/mL)		24,25$(OH)_2D_3$ (ng/mL)		25(OH)D_3/24,25$(OH)_2D_3$	
	r value	*P* value	*r* value	*P* value	*r* value	*P* value
Age (yr)	-0.034	0.711	-0.132	0.150	-0.134	0.145
ALT (U/L)	-0.062	0.502	0.175	0.055	-0.230	0.012
AST (U/L)	0.036	0.693	0.169	0.066	-0.149	0.104
TBIL (μmol/L)	-0.124	0.177	0.088	0.337	-0.176	0.054
DBIL (μmol/L)	-0.165	0.072	0.109	0.065	-0.282	0.002
TP (g/L)	-0.126	0.170	0.136	0.138	-0.185	0.043
ALB (g/L)	0.027	0.770	0.120	0.193	-0.112	0.223
GGT (U/L)	-0.143	0.119	0.104	0.260	-0.211	0.021
CHE (U/L)	-0.015	0.871	-0.058	0.526	-0.011	0.906
Ca (mmol/L)	0.027	0.768	0.200	0.028	-0.158	0.084
P (mmol/L)	0.088	0.337	-0.130	0.158	0.266	0.003
HB (g/L)	-0.040	0.665	0.133	0.149	-0.195	0.033
PTA (%)	0.109	0.237	-0.134	0.145	0.231	0.011
Liver fibrosis	-0.052	0.574	0.023	0.807	-0.072	0.435
Liver inflammation	-0.104	0.258	-0.129	0.160	0.069	0.451
Viral load(IU/mL)	0.074	0.464	0.173	0.086	-0.131	0.196
25(OH)D_3 (ng/mL)	-	-	0.410	< 0.001	-	-
24,25$(OH)_2D_3$ (ng/mL)	0.410	< 0.001	-	-	-	-

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TBIL: Total bilirubin; DBIL: Direct bilirubin; TP: Total protein; ALB: Albumin; GGT: Gamma-glutamyl transpeptidase; CHE: Cholinesterase; HB: Hemoglobin; PLT: Platelets; PTA: Prothrombin time activity.

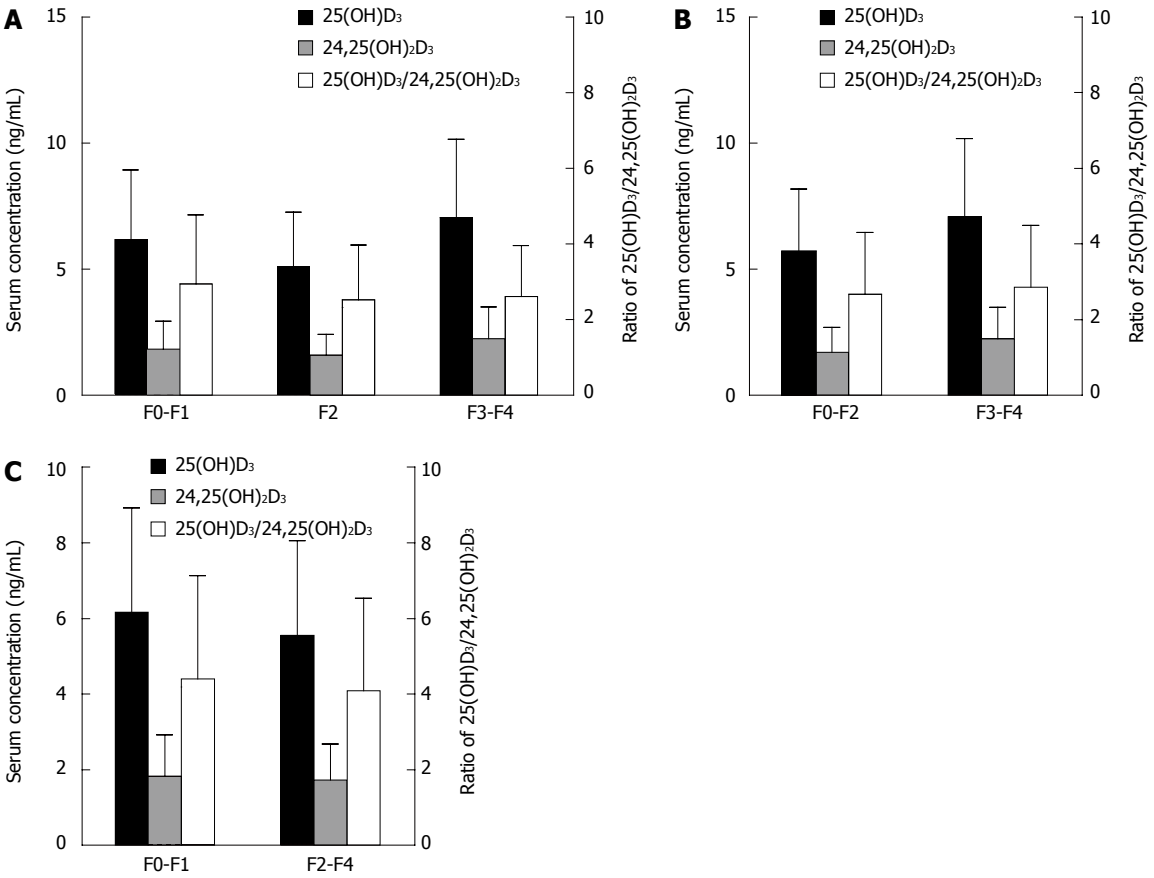

Figure 1 Serum vitamin D levels in chronic hepatitis C patients with different stages of fibrosis. Serum 25(OH)D_3 and 24,25$(OH)_2D_3$ levels, and the 25(OH)D_3/24,25$(OH)_2D_3$ ratio, among subgroups of patients with different stages of fibrosis. A: F0-F1 *vs* F2 *vs* F3-F4; B: F0-F2 *vs* F3-F4; C: F0-F1 *vs* F2-F4.

reasons for the inconsistencies are that the level of vitamin D can be affected by many factors, such as race, diet, altitude, light exposure, *etc*.[22], and that liver fibrosis is influenced by co-existing etiologies such as co-infection of HIV, antiviral treatment, and the method of measurement. The HPLC-MS/MS method

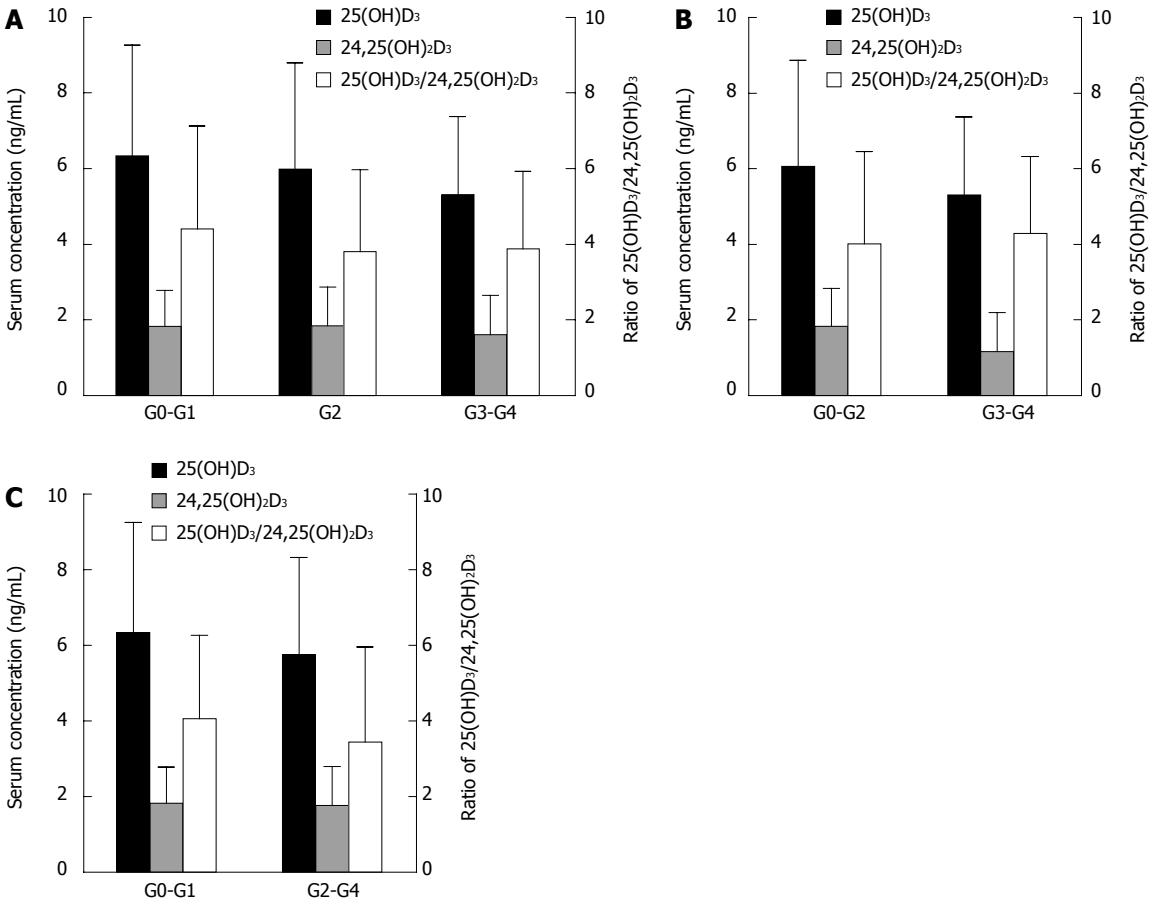

Figure 2 Serum vitamin D levels in chronic hepatitis C patients with different grades of liver inflammation. Serum 25(OH)D_3 and 24,25(OH)$_2D_3$ levels, and the 25(OH)D_3/24,25(OH)$_2D_3$ ratio, among subgroups of patients with different grades of liver inflammation. A: G0-G1 *vs* G2 *vs* G3-G4; B: G0-G2 *vs* G3-G4; C: G0-G1 *vs* G2-G4.

we employed is currently the best way to detect vitamin D, being more stable, reproducible, sensitive and accurate than other methods[23-26]. The use of HPLC-MS/MS in our study laid a solid foundation for investigating the relationship between vitamin D levels and liver fibrosis in patients with CHC.

Our results showed that CHC patients had lower serum 25(OH)D_3 levels than did healthy controls, which is consistent with previous findings[15,27]. In fact, the serum 25(OH)D_3 levels in our CHC patients were lower than the levels reported for CHC patients in previous studies. One reason for this difference might be that our patients were of Chinese Han ethnicity, not Caucasian. A recent study found serum 25(OH)D_3 levels in healthy Japanese adults to be 34.7 ± 16.4 nmol/L (13.88 ± 6.56 ng/mL), similar to our results[28]. Another reason might be the inadequate nutrition in our patients, due to their low income. We previously found that the mean body mass index (BMI) of our patients was 22.34 kg/m^2, which was classified as non-obese (BMI < 25 kg/m^2); the liver biopsies in the current study confirmed that 70.0% (84/120) of the patients did not have steatosis[29].

Based on liver biopsy, our study showed no association between serum 25(OH)D_3 levels and the degree of liver fibrosis in Chinese Han patients with CHC. This is consistent with the study of Kitson *et al*[9], in which HPLC-MS/MS was used to measure vitamin D_3.

Although serum 25(OH)D_3 levels also did not correlate with the grade of inflammation, we found that, from G0-G1 to G2 to G3-G4, the mean serum 25(OH)D_3 level changed from 6.35 ± 2.91 ng/mL to 5.99 ± 2.81 ng/mL to 5.31 ± 2.07 ng/mL. This did not exclude the effect of a small sample size in our study. Several studies have shown that severe liver inflammation can decrease 25-hydroxylase activity[9,30], thereby causing a decrease in 25(OH)D_3 levels. Further research is needed to elucidate the exact mechanism involved.

Furthermore, we measured serum 24,25(OH)$_2D_3$ levels and evaluated whether they were associated with liver fibrosis. Serum 24,25(OH)$_2D_3$ is the inactivated form of vitamin D. By measuring the level of 24,25(OH)$_2D_3$, we can infer whether there is excessive conversion of 25(OH)D_3 in patients with CHC. Although an obvious correlation exists between serum 25(OH)D_3 and 24,25(OH)$_2D_3$ levels, due to their metabolic connection, we found no difference in serum 24,25(OH)$_2D_3$ levels between CHC patients and healthy controls. In order to clarify the phenomenon, future studies should address whether the hydroxylase plays a key role in 24,25(OH)$_2D_3$ production.

Similar to serum 25(OH)D_3, neither serum 24,

$25(OH)_2D_3$ nor the ratio of $25(OH)D_3$ to $24,25(OH)_2D_3$ correlated with liver fibrosis or inflammation. The serum $24,25(OH)_2D_3$ levels did not differ among the subgroups of liver fibrosis or inflammation. To the best of our knowledge, this is the first report on serum $24,25(OH)_2D_3$ levels and the $25(OH)D_3/24,25(OH)_2D_3$ ratio in CHC patients.

There were some limitations in our study. We analyzed the relationship between serum vitamin D and hepatic fibrosis based on a long-term (about 20 years) follow-up in a cohort of treatment-naive CHC patients, and the actual number of patients was not large. The patient and control groups were not well-matched for age. Studies with larger group sizes are needed to verify our findings.

In conclusion, using liver biopsy and HPLC-MS/MS, we found that serum levels of $25(OH)D_3$ and its degradation metabolite $24,25(OH)_2D_3$ did not correlate with liver fibrosis or inflammation in treatment-naive Chinese Han CHC patients from the same geographical area with similar lifestyle and eating habits.

COMMENTS

Background

The liver plays an important role in vitamin D metabolism. Recent studies have found that there is a close relationship between vitamin D and hepatitis C. Although the serum levels of 25-hydroxyvitamin D_3 [$25(OH)D_3$] are known to be decreased in patients with chronic hepatitis C (CHC), the role of $25(OH)D_3$ in liver fibrosis is unclear. Correlation studies on serum $25(OH)D_3$ levels and liver fibrosis based on liver biopsy in Chinese Han patients with CHC have scarcely been reported.

Research frontiers

It is important to study the relationship between vitamin D and liver fibrosis in CHC patients. Several studies showed that the low $25(OH)D_3$ serum levels in patient with CHC were associated with the progression or degree of liver fibrosis and affected the virological response to anti-HCV therapy. In contrast, other studies found no correlation between serum $25(OH)D_3$ levels and liver fibrosis or sustained virological responses.

Innovations and breakthroughs

To the best of our knowledge, this is the first report evaluating the correlation between serum $24,25(OH)_2D_3$ levels, and the $25(OH)D_3/24,25(OH)_2D_3$ ratio, with liver fibrosis and inflammation in CHC patients.

Applications

The serum levels of $25(OH)D_3$ and its degradation metabolite $24,25(OH)_2D_3$ cannot be used to predict the extent of liver fibrosis or inflammation in treatment-naive Chinese patient with CHC.

Peer-review

The manuscript by Yan *et al* is a well-written manuscript with novel data.

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ORIGINAL ARTICLE

Retrospective Study

Prediction of the indication criteria for endoscopic resection of early gastric cancer

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Author contributions: Park JH, Park JM and Park CS contributed equally to this work; Park JH collected and analyzed the data, and drafted the manuscript; Park JM and Park CS provided analytical oversight; Lee SH designed and supervised the study; Park KS, Kim ES and Cho KB revised the manuscript for important intellectual content; all authors have read and approved the final version to be published.

Institutional review board statement: The study was reviewed and approved by the Yeungnam University hospital Institutional Review Board.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at e-mail: dr9696@nate.com.

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Abstract

AIM: To find risk factors of lymph node metastasis (LNM) in early gastric cancer (EGC) and to find proper endoscopic therapy indication in EGC.

METHODS: We retrospectively reviewed the 2270 patients who underwent curative operation for EGC from January 2001 to December 2008. EGC was defined as malignant lesions that do not invade beyond the submucosal layer of the stomach wall irrespective of presence of lymph node metastasis.

RESULTS: Among 2270 enrolled patients, LNM was observed in 217 (9%) patients. LNM in intramucosal (M) cancer and submucosal (SM) cancer was detected in 38 (2.8%, 38/1340) patients and 179 (19%, 179/930) patients, respectively. In univariate analysis, the risk factors for LNM in EGC were size of tumor, Lauren classification, ulcer, lymphatic invasion, vascular invasion, and depth of invasion. However, in multivariate analysis, size of tumor, lymphatic invasion, vascular invasion, and depth of invasion were risk factors for LNM in EGC. Size of tumor, lymphatic invasion, vascular invasion, and depth of invasion were risk factors for LNM in cases of intramucosal cancer and submucosal cancer. In particular, there was no lymph node metastasis in cases of well differentiated early gastric cancer below 1 cm in size without ulcer regardless of lymphovascular invasion.

CONCLUSION: Tumor size, perilymphatic-vascular invasion, and depth of invasion were risk factors for LNM in EGC. There was no LNM in EGC below 1 cm

regardless risk factors.

Key words: Early gastric cancer; Lymph node metastasis; Endoscopic resection

Core tip: Although the depth of tumor infiltration, tumor size as a maximum tumor diameter, and perilymphovascular invasion are independent risk factors for lymph node metastasis (LNM) in early gastric cancer (EGC), there was no LNM in intramucosal cancer which was not signet ring cell type and was below 1 cm without ulceration regardless of lymphatic invasion. This means that endoscopic submucosal dissection can be the treatment of choice in patients with intramucosal cancer below 1 cm without ulceration. There was LN metastasis in EGC of extended criteria in this study. But, the possibility of LNM in intramucosal cancer of extended indication was below 1%.

Park JH, Lee SH, Park JM, Park CS, Park KS, Kim ES, Cho KB. Prediction of the indication criteria for endoscopic resection of early gastric cancer. *World J Gastroenterol* 2015; 21(39): 11160-11167 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i39/11160.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i39.11160

INTRODUCTION

Early detection in gastric cancer is increasing with screening endoscopy. Consequently early gastric cancer (EGC) which was resectable with endoscopic resection has increased. In gastric cancer, the most significant factor in endoscopic resection is the absence of lymph node metastasis (LNM) because it determines the treatment. For that reason, prediction of lymph node metastasis in EGC is very important.

The predictors of the absence of LNM in EGC were tumor size of 2 cm or smaller, histologically differentiated type, intramucosal cancer, and no lymphovascular (LV) invasion[1]. According to the risk factors of LNM, endoscopic submucosal dissection (ESD) is a standard treatment for differentiated-type adenocarcinoma without ulceration, of which the depth of invasion is up to muscularis mucosa and the diameter is below 2 cm (Japanese gastric cancer treatment guidelines 2010[1]). However, some recent studies have reported extended indications for endoscopic resection[2-6] in differentiated EGC without lymphatic or vascular involvement, including: (1) mucosal cancers with no ulcerative findings, regardless of tumor size; (2) mucosal cancers with ulcerative findings $\leqslant$ 30 mm; and (3) minute ($\leqslant$ 500 μm from the muscularis mucosae) submucosal invasive cancers $\leqslant$ 30 mm.

However, evidence from these studies is limited in South Korea. Thus, the purpose of this study was to determine the risk factors of lymph node metastasis in EGC removed by gastrectomy and to determine the safety of extended criteria for endoscopic treatment of EGC in South Korea.

MATERIALS AND METHODS

A total of 2270 patients who had undergone gastrectomy with lymph node dissection for EGC at Yeungnam University hospital and Keimyung University hospital and we retrospectively reviewed the patient who has been taken radiologic imaging study and upper gastrofibroscope, and confirmed pathological reports after operation.

The patient profiles were investigated, including sex, age, tumor location, size, ulceration, histological type, lymphovascular invasion, and depth of invasion. Well and moderately differentiated tubular adenocarcinoma and papillary adenocarcinoma were classified as differentiated lesions. Poorly differentiated adenocarcinoma, signet ring cell carcinoma, and mucinous carcinoma were categorized as undifferentiated types. Lesions with ulcer or ulcer scar within cancer were regarded as ulcerated lesions. The depth of submucosal invasion was checked from the muscularis mucosa to the point of deepest penetration. The depth of submucosal invasion was subclassified according to two groups: SM1 ($\leqslant$ 500 μm penetration into submucosa) and SM2 (> 500 μm). The tumor size was measured by the results of the pathological report after surgical resection.

Statistical analysis was performed using the SPSS program. The relationship between lymph node metastasis and various factors was assessed using the simple χ^2 test and multiple logistic regression analysis.

RESULTS

Baseline characteristics of enrolled patients and EGC

Male to female ratio was 1488:782 and mean age was 59.3 ± 11.7. The mean length of major axis was 25.9 ± 16.5 mm. The most common location of early gastric cancer was middle anterior wall of stomach.

Risk factors of LNM

Among 1340 patients with M cancers, 2.8% (39/1340) were diagnosed as LMN; 19.2% (179/930) in SM cancer, 14.5% (55/379) in SM1 lesion, and 22.3% (123/551) in SM2 lesion. The relationships between various clinical or histological factors and the risk of LNM are summarized in Table 1, Table 2 and Table 3. Tumor size, lymphatic or venule invasion, deeper vertical invasion, and ulceration were the risk factors of lymp node metastasis. Similar to the finding for cancer involving intramucosa, significant correlation was observed between tumor larger than 3 cm and

Table 1 Risk factors for lymph node metastasis in early gastric cancer (n = 2270) by multivariate analysis

	OR	95%CI	*P* value
Tumor size ⩽ 30 mm *vs* > 30 mm	2.1	1.5-3.0	< 0.001
Depth of invasion			
M *vs* SM1	1.6	1.0-2.4	< 0.001
M *vs* SM2	4.7	3.0-7.2	0.040
Lymphatic invasion	4.1	2.8-6.0	< 0.001
Vascular invasion	4.7	3.1-7.1	< 0.001

Table 2 Risk factors for lymph node metastasis in intramucosal gastric cancer (n = 1340) by univariate analysis n (%)

	Negative	Positive	*P* value
Gender			0.217
Male/female	843/458	29/10	
Age	58.5 ± 11.8	50.6 ± 14.5	< 0.001
Size of tumor (mm, mean ± SD)	23.5 ± 15.7	37.9 ± 28.6	< 0.001
Tumor size			< 0.001
⩽ 30 mm	961 (81)	18 (54)	
> 30 mm	224 (19)	15 (46)	
Tumor location			0.052
Upper	192 (15)	14 (36)	
Middle	736 (59)	19 (48)	
Lower	324 (26)	6 (16)	
Lauren			< 0.001
Intestine type	634 (61)	11 (38)	
Diffuse type	371 (36)	16 (55)	
Mix type	33 (3)	2 (7)	
Histologic type			0.715
Differentiated	799 (62)	20 (53)	
Undifferentiated	498 (38)	18 (47)	
Ulcer finding			0.757
Absence/presence	619/550	14/14	
Lymphatic invasion			< 0.001
Absence/presence	1202/93	23/15	
Vascular invasion			< 0.001
Absence/presence	1272/22	22/17	
Perineural invasion			< 0.001
Absence/presence	1279/13	36/3	

lymphovascular invasion with an increased risk of LNM. Also, cancer with involvement deep into the submucosa showed greater association with LNM; and significant correlation was observed between tumor larger than 2 cm and lymphovascular invasion and LNM in submucosal cancer. This meant that the possibility of LNM in cancer with involvement deep into the submucosa was greater than in smaller sized tumor than in intramucosal cancer.

Suggestion for expanded indication of endoscopic resection for EGC

According to extended criteria, 3 (0.8%, 3/378) differentiated intramucosal lesions without lymphovascular invasion and ulceration regardless of tumor size showed association with LNM. Two (0.9%, 2/230) differentiated intramucosal ulcerative lesions below 3 cm without lymphovascular invasion showed association with LNM. Three (2.7%, 3/113)

Table 3 Risk factors for lymph node metastasis in submucosal gastric cancer (n = 930) by univariate analysis n (%)

	Negative	Positive	*P* value
Gender			0.369
Male/female	497/254	119/60	
Age	61.0 ± 11.3	60.2 ± 11.4	< 0.001
Size of tumor (mm, mean ± SD)	27.0 ± 14.8	36.1 ± 19.9	< 0.001
Tumor size			< 0.001
⩽ 20 mm	512 (73)	94 (55)	
> 20 mm	188 (27)	76 (45)	
Tumor location			0.651
Upper	157 (21)	34 (20)	
Middle	376 (50)	93 (52)	
Lower	223 (29)	52 (28)	
Lauren			0.514
Intestine type	343 (56)	69 (47)	
Diffuse type	215 (35)	63 (43)	
Mix type	52 (9)	16 (10)	
Histologic type			0.884
Differentiated	489 (65)	113 (63)	
Undifferentiated	258 (35)	66 (37)	
Ulcer finding			0.115
Absence/presence	255/388	51/104	
Lymphatic invasion			< 0.001
Absence/presence	526/222	47/130	
Vascular invasion			< 0.001
Absence/presence	665/82	104/71	
Perineural invasion			0.103
Absence/presence	693/56	157/22	

differentiated submucosal (⩽ 500 μm from the muscularis mucosae) lesions were below 3 cm without lymphovascular invasion (Table 4). Although there were few patients with LNM in cases reflected by extended criteria, the possibility of LNM in EGC remained.

In our study, none (0/102) of the differentiated intramucosal lesions below 1 cm without lymphovascular invasion and ulceration showed association with LNM. In particular, there was no lymph node metastasis (0/127) in cases of well differentiated early gastric cancer below 1 cm in size without ulcer regardless of lymphovascular invasion. The undifferentiated intramucosal cancer below 1 cm in size with ulcer did not show association with metastasis. One (1.2%, 1/81) of the undifferentiated early gastric cancers below 1 cm without ulcer regardless of lymphovascular invasion showed association with LNM. The cell type of the patient in one case was signet ring. Thus, there was no LNM in patients with early gastric cancer below 1 cm in the without ulcer group without signet ring type cancer in cell differentiation (Table 4).

Relative contraindication of endoscopic resection for EGC

Of 406 patients with undifferentiated M cancer, 2.7% (11/406) were found to have LNM. Seven patients with perilymphatic-vascular invasion were confirmed LNM and four of them had no ulcerated lesion. In 11 patients with LNM, one case was below 10 mm in size (Table 4).

Table 4 Lymph node metastasis according to the presence of ulcer, differentiation, lymphovascular invasion, and the depth of invasion in early gastric cancer patients

Ulcer	Differentiation	VI	LI	Size			
				≤ 10 mm	> 10 mm	> 20 mm	> 30 mm
M							
Ulcer negative	Differentiated	No	No	0/102	2/112	0/81	1/57
			Yes	0/5	0/2	0/1	0/5
		Yes	No	0	0	0	1/2
			Yes	0	1/2	0/0	1/2
	Undifferentiated	No	No	1/66	2/41	0/41	1/45
			Yes	0/3	0/3	0/1	0/2
		Yes	No	0	0	0	0
			Yes	0	0	0/1	3/3
Ulcer positive	Differentiated	No	No	1/59	1/104	0/67	1/47
			Yes	0/5	1/12	0/1	1/5
		Yes	No	0	0	0	0
			Yes	0/2	0/4	0/0	1/1
	Undifferentiated	No	No	0/40	0/50	1/42	0/46
			Yes	0/4	0/2	0/6	1/4
		Yes	No	0	0	0	0
			Yes	0/1	1/2	0/1	1/1
SM1							
Ulcer negative	Differentiated	No	No	0/10	1/19	0/12	1/13
			Yes	0/3	0/3	1/2	0/1
		Yes	No	0	0	1/1	2/2
			Yes	0	1/3	0/0	1/3
	Undifferentiated	No	No	0/7	0/6	1/8	0/6
			Yes	0	0/2	0/0	0/1
		Yes	No	0	0	0	0
			Yes	0	0/1	0/1	2/3
Ulcer positive	Differentiated	No	No	0/14	2/24	0/19	1/13
			Yes	0/1	0/0	1/7	1/5
		Yes	No	0	0/1	0/0	0
			Yes	0	0/1	2/3	3/3
	Undifferentiated	No	No	0/6	2/12	2/7	1/6
			Yes	0/2	1/7	0/3	1/7
		Yes	No	0	0	0	1/1
			Yes	1/2	3/5	1/4	1/1
SM2							
Ulcer negative	Differentiated	No	No	0/4	2/23	1/25	0/18
			Yes	0	1/12	2/5	11/27
		Yes	No	0/1	0/0	0/0	0
			Yes	0/1	1/3	0/1	6/8
	Undifferentiated	No	No	0/1	1/14	2/9	1/4
			Yes	0/2	1/1	0/2	3/7
		Yes	No	0/1	0/0	0/0	0
			Yes	0/1	0/0	5/6	1/3
Ulcer positive	Differentiated	No	No	3/16	2/33	1/31	3/31
			Yes	0/3	5/15	12/27	13/31
		Yes	No	0	0	1/3	0/3
			Yes	0/1	1/2	2/5	4/5
	Undifferentiated	No	No	0/4	2/14	1/19	4/18
			Yes	0/2	1/8	1/6	4/11
		Yes	No	0	0	0/1	1/1
			Yes	1/1	3/4	5/8	3/5

In 324 patients with undifferentiated submucosal cancer, 20.3% (66/324) were found to have LNM. Of the subgroup of 16 patients with undifferentiated SM1 lesion, 16.3% (16/98) were diagnosed as LNM. Yet, of 82 undifferentiated SM1 lesions without LNM, 31 cases had ulcerated lesion, no one had perivascular invasion, 32 cases showed perilymph invasion. And, of 16 undifferentiated SM1 lesions with LNM, no one had perivascular invasion, and perilymph invasion was detected in three cases. Seven patients had lymphatic and vascular invasion.

Based on these results, the treatment of choice of

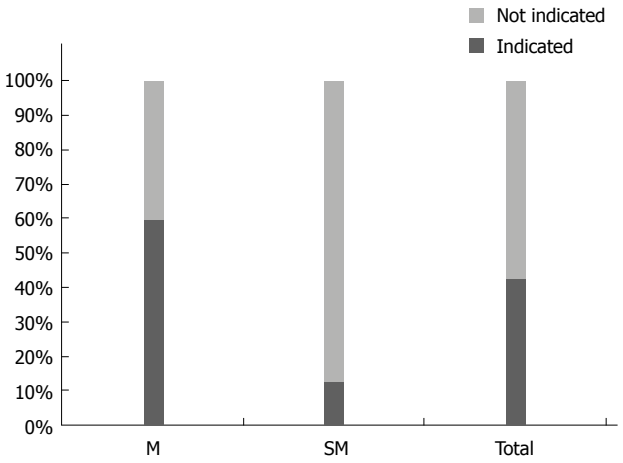

Figure 1 Percentage of cases in which endoscopic resection was indicated among early gastric cancer patients who underwent surgery.

undifferentiated SM1 lesion is not endoscopic resection but surgical resection (Table 4).

Percentage of cases in which endoscopic resection was indicated among EGCa patients who underwent surgery

In Yeungnam and Keimyung university hospitals, 1340 patients with M cancer underwent surgery. Among them, 799 (59.6%) patients were confirmed as differentiated M cancer without LNM. Among 930 patients with SM invasion, 118 (12.6%) differentiated SM1 cancer patients without perilymphatic-vascular invasion were free from LNM; 40.3% (917/2270) of patients with EGC were overtreated (Figure 1).

DISCUSSION

The definition of EGC was suggested by the Japanese Gastroenterologic Endoscopic Society in 1962. In this definition, EGC is defined as gastric cancer which invades within submucosa regardless of lymph node metastasis[7].

The existence of LNM was association with bad prognosis[8]. The 5-year survival rate is > 90% in EGC, and the absence of lymph node metastasis is the most significant prognostic factor[9,10]. The 5-year survival rate was reported to be 87.3% in patients with regional lymph nodes metastasis and 94.2% in those without[11]. Maruyama *et al*[12] reported that the number of metastatic lymph nodes in patients with EGC was associated with the survival rate. EGC patients with LNM had a lower survival rate than patients without lymph node metastasis[13].

Because of an increased accuracy of diagnosis of EGC, which in turn leads to a better prognosis, increased interest has been focused on betterment of the quality of life and minimalization of invasive procedures. Furthermore, nowadays, various minimally invasive treatment modalities have been developed and endoscopic resection has enabled rapid restoration of patient's health with lower risk of procedure, however, this endoscopic treatment also has risk of disease recurrence and distant metastasis.

Therefore, we suggested investigation of the relationship between various risk factors and LNM, and we think that our research will be helpful in development of more delicate criteria for endoscopic resection of EGC.

The overall incidence of a LNM in EGC ranges from 10% to 15%[9,10,14,15]. The incidence of lymph node invasion was reported upto 4.8% in mucosal cancers and 23.6% in SM cancers[9,10,16]. These results are similar to ours generally, and the incidence of LNM was similar to those in mucosal carcinoma reported by Yamao *et al*[17] and Tsujitani *et al*[18], and lower risk in submucosal carcinoma reported by other researchers[19].

So far, standard treatment of SM cancer is gastrectomy with lymphadenectomy[20]. But, endoscopic resection such as EMR or ESD is widely used standard treatment modality for mucosal cancer[9,21]. In studies analyzing the outcome of endoscopic resection in undifferentiated EGC, complete resection rate of undifferentiated EGC was relatively lower than that of differentiated EGC[22].

There were various attempts to clarify risk factors predicting LNM and relationship between these risk factors and prognosis. Maehara *et al*[23] found that large tumor, lymphatics involvement, and submucosal invasion were risk factors for LNM in EGC patients. Yamao *et al*[17] also reported that lymphatic invasion, histologic type, and large tumor size were independent risk factors for LNM in intramucosal EGC. In our study, univariate and multivariate analysis showed association of LNM in EGC with large tumor size, submucosal invasion, and perilymphatic-vascular invasion.

Some researchers have advocated that submucosal invasion is the most predictive risk factor for LNM. Otherwise, of risk factors for metastasis, the most important is perilymphatic invasion[24]. As for lymphatic vascular invasion, this may be the most important direct route to the regional lymph nodes[6]. Relation of prognostic factors, including gender, tumor size, depth of invasion, endoscopic findings, and lymphatic invasion to LNM in SM cancer has been demonstrated[20,25,26]. Besides pathologic type, Lauren classification and perineural involvement also showed significant and independent association with LNM.

However, Keita Nakahara *et al*[6] reported that the histological type was not a risk factor for LNM. In our results, histological type and presence of differentiation were unrelated to LNM. And, in the study by Nakahara *et al*[6], no significant difference in invasion depth was observed between SM1 and M cancers among EGC. Yet, in our studies, a statistically significant difference was observed between SM1 and M, and SM1 and SM2. With respect to size and ulceration, the possibility of SM invasion is higher and ulceration develops more readily in larger lesions. In contrast, there was no difference between ulcerated lesion and LNM in

M, SM1, and SM2 (Table 1). Molecular biologically, proliferating cell nuclear antigen labeling index of greater than 25%, matrix metalloproteinase-9-positive tumors, tumor with gastric mucin phenotype, and vascular endothelial growth factor-C-positive tumors showed an association with LNM[27].

EMR has been widely used for treatment of EGC. However, current application of EMR is limited to differentiated EGC[28]. Endoscopic resection is basically contraindicated even in differentiated type submucosal gastric cancer. In fact, Kunisaki *et al*[29] reported that the incidence of LNM was 1.8% in patients with submucosal gastric cancer measuring less than 20 mm and without lymphovascular invasion, and the site of LNM was restricted to the paragastric lymph nodes.

Kurihara *et al*[19] reported that reoperation after an endoscopic resectio for EGC with SM1 invasion is unnecessary because most SM1 cancers < 20 mm do not have the lymph node metastasis. Gotoda *et al*[4] reported that none of 145 differentiated adenocarcinomas < 30 mm, a lack of lymphovascular invasion and submucosal penetration < 500 μm, had nodal metastasis[4]. Gotoda *et al*[4] proposed an extension of the indications for endoscopic treatment, and one of the extended indications was differentiated SM1 adenocarcinoma measuring less than 30 mm[4]. Abe *et al*[30] also reported that an EMR could be suitable for SM1 cancers and no lymphatic invasion. Abe *et al*[30] suggested that SM cancer with < 15 mm in diameter could be treated by EMR or a local resection. In our study, there were few patients with LNM in cases reflected by extended criteria. However, the possibility of LNM in EGC remained. In particular, there was no LNM in patients with EGC below 1 cm without ulcer in the group without signet ring type cancer in cell differentiation (Table 4).

Korenaga *et al*[31] reported that accurate assessment of depth of cancer invasion was difficult in lesions larger than 15 mm resected piecemeal. Clinically, it is difficult to determine whether lesions are confined to the mucosa or not[32,33]. Also, several studies reported that assessment of depth of cancer invasion after endoscpic resection is inaccurate in up to 20% of lesions[32,33]. Biopsies are too superficial to provide this information, but EMR provides a larger specimen, which allows assessment of depth of cancer invasion and lymphovascular invasion[4]. But, when resection if fragmented, the bruised margin makes it difficult to evaluate the stump, and the degree of radical treatment cannot be adequately evaluated. This issue is particularly important in lesions for which local treatment is indicated. When en bloc/total resection is technically impossible, indications of EMR should not be readily extended[6]. In addition, accurate evaluation of the presence of lymphatic vascular invasion preoperatively or before endoscopic resection is impossible, therefore, postoperative histological evaluation is essential.

Endoscopic ultrasonography, computed tomography *etc*. were widely used by staging of EGC. But, resected tissue by EMR or ESD and radiological examination could not exclude perfectively regional LNM. However, recently, minimal invasive surgery or stomach conserving therapy such as sentinel node navigation surgery, hybrid NOTES, and endoscopic submucosal dissection with sentinel node navigation surgery has been newly developed[34,35].

In addition, from the technical perspective of en block/total resection, a condition of 30 mm or less may be considered safe[6]. The new endoscopic technique or tools such as ESD and IT knife enable complete resection of large and ulcerated lesions.

Because of the high probability of LNM and tumor residual, patients with submucosal undifferentiated EGC are not candidates for treatment by EMR[28]. By our studies, 66 (20.3%) cases of 324 undifferentiated SM cancer were related to LNM.

In the Kunisaki *et al*[36] study, the incidence of LNM in patients with poorly differentiated type mucosal cancer was 2.2%. In this study, the incidence of LNM was 3.4% in patients with undifferentiated mucosal tumors. Therefore, endoscopic resection may be contraindicated in these patients.

As mentioned above, currently, exclusion of histologically poorly differentiated submucosal gastric cancer from the indications for endoscopic resection is mandatory, whereas histologically differentiated submucosal gastric cancer can be curatively resected endoscopically[36]. However, endoscopic resection may be reasonable for histologically undifferentiated mucosal gastric cancer below 10 mm in size and without lymphovascular invasion, for undifferentiated submucosal cancer less than 10 mm and without lymphovascular invasion in elderly patients with severe co-morbid disease, because the incidence of LNM is low in these patients.

In conclusion, we suggest that depth of tumor infiltration, tumor size as a maximum tumor diameter, and perilymphovascular invasion are independent risk factors for LNM in EGC.

Our study shows that there was no LNM in intramucosal cancer which was not signet ring cell type and was below 1 cm without ulceration regardless of lymphatic invasion. This means that ESD can be the treatment of choice in patients with intramucosal cancer below 1 cm without ulceration.

ESD has shown advantages over conventional EMR for removal of larger or ulcerated EGC lesions in an *en bloc* manner as well as for prevention of residual disease and local recurrence. Some reports showed LN metastasis in EGC of extended indication, particularly in submucosal invasive EGC. Our study shows LN metastasis in EGC of extended criteria, too. But, the possibility of LNM in intramucosal cancer of extended indication was below 1%. Many more cases of materials and more large scaled multicenter studies are essential for development of eligibility criteria for endoscopic treatment of EGC.

COMMENTS

Background

Recently endoscopic resection of early gastric cancer (EGC) has been widely performed. In endoscopic treatment, the presence of lymph node metastasis (LNM) is the most important issue. However, data on risk factors for LNM in South Korean EGC have been limited. The aims of this study were to find risk factors of LNM in EGC and to find proper endoscopic therapy indication in EGC.

Research frontiers

According to the risk factors of LNM, endoscopic submucosal dissection (ESD) is a standard treatment for differentiated-type adenocarcinoma without ulceration, of which the depth of invasion is up to muscularis mucosa and the diameter is below 2 cm. However, recent studies have reported extended indications for endoscopic resection in differentiated EGC with no lymphatic or vascular involvement, including: (1) mucosal cancers without ulcerative findings, regardless of tumor size; (2) mucosal cancers with ulcerative findings ⩽ 30 mm; and (3) minute (⩽ 500 μm from the muscularis mucosae) submucosal invasive cancers ⩽ 30 mm. However, evidence from these studies is limited in South Korea.

Innovations and breakthroughs

Although the depth of tumor infiltration, tumor size as a maximum tumor diameter, and perilymphovascular invasion are independent risk factors for LNM in EGC, there was no LNM in intramucosal cancer which was not signet ring cell type and was below 1 cm without ulceration regardless of lymphatic invasion. This means that ESD can be the treatment of choice in patients with intramucosal cancer below 1 cm without ulceration. Gotoda *et al* proposed an extension of the indications for endoscopic treatment, and one of the extended indications was histologically differentiated type SM1 tumor measuring less than 30 mm. But, there was LN metastasis in EGC of extended criteria in this study. However, the possibility of LNM in intramucosal cancer of extended indication was below 1%.

Applications

There was no LNM in intramucosal cancer which was not signet ring cell type and was below 1 cm without ulceration regardless of lymphatic invasion. This means that ESD can be the treatment of choice in patients with intramucosal cancer below 1 cm without ulceration.

Terminology

The depth of submucosal invasion was subclassified according to two groups: SM1 (⩽ 500 μm penetration into submucosa) and SM2 (> 500 μm). Extended indication criteria for endoscopic resection in differentiated EGC with no lymphatic or vascular involvement, includes: (1) mucosal cancers without ulcerative findings, regardless of tumor size; (2) mucosal cancers with ulcerative findings ⩽ 30 mm; and (3) minute (⩽ 500 μm from the muscularis mucosae) submucosal invasive cancers ⩽ 30 mm.

Peer-review

The present paper is certainly well designed and conducted and draws solid and convincing conclusion that are mostly in line with similar studies from recent literature.

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ORIGINAL ARTICLE

Clinical Trials Study

Prognostic significance of plasma interleukin-6/-8 in pancreatic cancer patients receiving chemoimmunotherapy

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Informed consent statement: All study participants, or their legal guardian, provided informed written consent prior to study enrollment. The procedures were performed in accordance with the Helsinki Declaration.

Conflict-of-interest statement: Okamoto M holds ownership interest in Tella, Inc. Sugiyama H is the inventor of patents PCT/JP02/02794 and PCT/JP04/16336 which are held by the International Institute of Cancer Immunotherapy. No potential conflicts of interest were disclosed by the other authors.

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Abstract

AIM: To investigate the association of plasma levels of interleukin (IL)-6 and -8 with Wilms' tumor 1 (WT1)-specific immune responses and clinical outcomes in patients with pancreatic ductal adenocarcinoma (PDA) treated with dendritic cells (DCs) pulsed with three types of major histocompatibility complex class Ⅰ and Ⅱ-restricted WT1 peptides combined with chemotherapy.

METHODS: During the entire treatment period, plasma levels of IL-6 and -8 were analyzed by ELISA. The induction of WT1-specific immune responses was assessed using the WT1 peptide-specific delayed-type hypersensitivity (DTH) test.

RESULTS: Three of 7 patients displayed strong WT1-DTH reactions throughout long-term vaccination with significantly decreased levels of IL-6/-8 after vaccinations compared with the levels prior to treatment. Moreover, overall survival (OS) was significantly longer in PDA patients with low plasma IL-6 levels (< 2 pg/mL) after 5 vaccinations than in patients with high plasma IL-6 levels (⩾ 2 pg/mL) (P = 0.025). After disease progression, WT1-DTH reactions decreased severely and were ultimately negative at the terminal stage of cancer. The decreased levels of IL-6/-8 observed throughout long-term vaccination were associated with WT1-specific DTH reactions and long-term OS.

CONCLUSION: Prolonged low levels of plasma IL-6/-8 in PDA patients may be a prognostic marker for the clinical outcomes of chemoimmunotherapy.

Key words: Chemoimmunotherapy; Dendritic cell; Delayed-type hypersensitivity; Interleukin-6; Interleukin-8; Pancreatic cancer; Wilms' tumor 1

Core tip: We recently reported a phase 1 clinical study in pancreatic cancer patients using dendritic cells (DCs) pulsed with multiple major histocompatibility complex class Ⅰ and Ⅱ-restricted Wilms' tumor 1 (WT1) epitopes (DC/WT1-Ⅰ/Ⅱ) in combination with chemotherapy. Little is known about the prognostic markers for the clinical outcomes of chemoimmunotherapy. We examined the association of plasma levels of interleukin (IL)-6/-8 with WT1-specific immune responses and clinical outcomes in pancreatic cancer patients treated with chemotherapy combined with DC/WT1-Ⅰ/Ⅱ. The study demonstrates that prolonged low levels of plasma IL-6/-8 in pancreatic ductal adenocarcinoma patients may be a prognostic marker for the clinical outcomes of chemoimmunotherapy.

Tsukinaga S, Kajihara M, Takakura K, Ito Z, Kanai T, Saito K, Takami S, Kobayashi H, Matsumoto Y, Odahara S, Uchiyama K, Arakawa H, Okamoto M, Sugiyama H, Sumiyama K, Ohkusa T, Koido S. Prognostic significance of plasma interleukin-6/-8 in pancreatic cancer patients receiving chemoimmunotherapy. *World J Gastroenterol* 2015; 21(39): 11168-11178 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i39/11168.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i39.11168

INTRODUCTION

Patients with pancreatic ductal adenocarcinoma (PDA) have a particularly poor prognosis, with a 5-year survival rate of < 1%[1]. Thus, PDA remains one of the deadliest human tumors, characterized by high mortality, rapid progression, and resistance to chemotherapy and radiation therapy. Compared with single chemotherapy with standard agents such as gemcitabine, multi chemotherapy regimens such as FOLFIRINOX (consisting of 5-fluorouracil/folinic acid/oxaliplatin/irinotecan) and gemcitabine/nab-paclitaxel have been associated with significant improvement in median overall survival (OS) from 6 to 11 mo[2,3]. New therapeutic approaches for PDA are urgently needed. PDA cells express tumor-associated antigens (TAAs), including Wilms' Tumor gene 1 (WT1)[4]. Therefore, immunotherapy targeting PDA-associated antigens may be an alternative approach in patients with PDA.

Dendritic cells (DCs) are potent antigen-presenting cells (APCs) extensively used for the development of anticancer immunotherapies[5,6]. DCs capture and process TAAs into peptides and present these fragments through major histocompatibility complex (MHC) class Ⅰ and Ⅱ pathways, thus simultaneously stimulating both $CD4^+$ and $CD8^+$ T cells[5,6]. TAAs are recognized by $CD8^+$ cytotoxic T-lymphocytes (CTLs) in the context of MHC class Ⅰ molecules, whereas $CD4^+$ T cells recognize antigenic peptides in association with MHC class Ⅱ molecules. $CD8^+$ CTLs recognize MHC class Ⅰ-peptide complexes on cancer cells and destroy these cells through effector molecules such as granzyme B and perforin[7]. DCs have been pulsed with various MHC class Ⅰ-restricted antigenic peptides for the treatment of patients with PDA in clinical studies; however, the antitumor effects of these vaccines targeting only $CD8^+$ CTLs are not as vigorous in clinical trials[7]. Increasing evidence has suggested that $CD4^+$ T cells prime and maintain antigen-specific $CD8^+$ CTLs[8] and play a direct role in the destruction of tumor cells[9]. Therefore, the stimulation of both $CD4^+$ and $CD8^+$ T cells is an efficient strategy for treating patients with advanced cancer. We recently conducted a phase 1 clinical study in patients with PDA to examine the clinical and immunological responses to DCs pulsed with

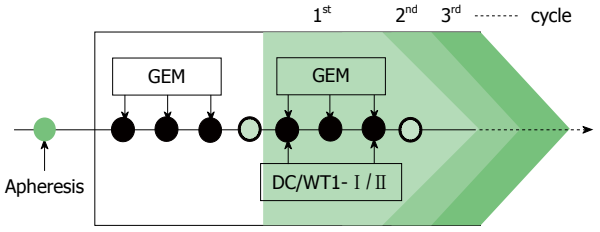

Figure 1 Treatment schedule for patients with pancreatic ductal adenocarcinoma. The patients were treated with gemcitabine alone, followed by dendritic cells pulsed with a Wilms' tumor 1 (WT1)-specific peptide mixture restricted by multiple major histocompatibility complex (MHC) class Ⅰ and Ⅱ molecules (DC/WT1-Ⅰ/Ⅱ) in combination with gemcitabine or S-1, an oral 5-fluorouracil (5-FU). GEM: Gemcitabine.

multiple MHC class Ⅰ and Ⅱ-restricted WT1 epitopes (DC/WT1-Ⅰ/Ⅱ) in combination with chemotherapy[10,11]. The vaccination of PDA patients with DC/WT1-Ⅰ/Ⅱ simultaneously induced WT1-specific $CD4^+$ and $CD8^+$ T cell responses *in vivo* and *in vitro*[10,11]. WT1-specific delayed-type hypersensitivity (DTH) induced by combination therapy was associated with maintenance of WT1-specific memory CTLs, resulting in long-term clinical responses[10]. Moreover, we previously reported that the post-treatment neutrophil to lymphocyte (N/L) ratio is a treatment-related prognostic factor for improved survival of PDA patients after DC/WT1-Ⅰ/Ⅱ treatment[11]. In the present study, we analyzed the association of plasma interleukin (IL)-6 and IL-8 levels and WT1 peptide-specific DTH in PDA patients during long-term chemoimmunotherapy using DC/WT1-Ⅰ/Ⅱ. The value of plasma IL-6 and IL-8 levels as prognostic markers was also assessed.

MATERIALS AND METHODS

Study design

The study was reviewed and approved by the ethics committee of the Jikei Institutional Review Board, Jikei University School of Medicine (Tokyo, Japan), and the clinical study committee of Jikei University Kashiwa Hospital [No. 14-60 (3209) and 21-204 (6082)]. All 7 PDA patients provided written informed consent and underwent chemoimmunotherapy with DC/WT1-Ⅰ/Ⅱ vaccination and chemotherapy[10]. All procedures were performed in accordance with the Helsinki Declaration.

DC/WT1-Ⅰ/Ⅱ vaccine

Autologous peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood by Ficoll-Plaque Premium (GE Healthcare Bio-Sciences, Piscataway, NJ, United States) density gradient solution. Adherent PBMCs were cultured for 5 d in AIM-V medium (Gibco Life Technologies, New York, United States) containing granulocyte macrophage colony-stimulating factor (50 ng/mL, Primmune Corp. Kobe, Japan) and IL-4 (50 ng/mL, R&D Systems, Minneapolis, MN, United States) to generate immature DCs. The immature DCs were activated with penicillin-killed and lyophilized preparations of a low-virulence strain (Su) of Streptococcus pyogenes (OK-432; 10 mg/mL, Chugai Pharmaceutical Co, Ltd, Tokyo, Japan) and prostaglandin E2 (PGE2; 50 ng/mL, Daiichi Fine Chemical Co, Ltd, Toyama, Japan) for an additional 24 h. The mature DCs were pulsed with a mixture of three WT1 peptide types restricted to HLA-A*02:01, A*02:06 (126-134: RMFPNAPYL), A*24:02 (235-243: CYTWNQMNL) and MHC-class Ⅱ (332-347: KRYFKLSHLQMHSRKH; NeoMPS Inc., San Diego, CA, United States) as previously described[10].

Chemoimmunotherapy

Gemcitabine was intravenously administered at a dose of 1000 mg/m^2 on days 1, 8, and 15 of a 28-d cycle. After the first cycle of gemcitabine administration, the patients were treated with a combination of gemcitabine and DC/WT1-Ⅰ/Ⅱ. The DC/WT1-Ⅰ/Ⅱ vaccine (usually 1 × 10^7 cells/dose) was intradermally administered biweekly, regardless of the chemotherapy regimen (Figure 1). In Japan, the oral 5-fluorouracil (FU) S-1 is used to treat patients with gemcitabine-refractory PDA[12]. Therefore, some patients with gemcitabine-refractory PDA received S-1 during chemoimmunotherapy. Patients without early progressive disease received chemoimmunotherapy until the occurrence of disease progression, unacceptable adverse events, or withdrawal of patient consent.

Clinical responses

Computed tomography was performed every 4 to 8 wk during treatment until disease progression. The clinical response was determined according to Response Evaluation Criteria in Solid Tumors. Stable disease (SD) was defined as disease that was stable for more than 8 wk after the start of treatment. Overall survival (OS) and progression-free survival (PFS) were calculated from the date of treatment to the date of death or final follow-up and the date of disease progression, respectively.

WT1 peptide-specific immune responses

To determine the induction of WT1-specific immune responses during chemoimmunotherapy, the WT1 peptide-specific DTH test was performed before treatment and after every vaccination. Briefly, 30 μg of the three types of WT1 peptides in saline or saline alone was intradermally injected separately into the forearm. The maximum diameter of erythema and induration were measured 48 h after WT1 peptide injection. WT1-specific DTH positivity was defined as erythema and induration greater than 2 mm in the maximum diameter. We selected the value of 5-mm erythema and induration to discriminate between weak (2-5 mm) and strong (> 5 mm) WT1-specific DTH reactions. The DTH test was performed prior to treatment and during long-term treatment.

Table 1 Patient characteristics

No.	Sex	Age (yr)	Location	Size (mm)	Metastases	UICC stage	Vaccine (times)	OS (d)	PFS (d)	HLA type						Best overall tumor response
										HLA-A		DRB1		DPB1		
1	M	70	Body	22	Peritonitis	Ⅳ	35	582	440	02:01	24:02	04:05	15:02	05:01	09:01	Stable disease
2	M	68	Body	15	Liver, lymph nodes	Ⅳ	46	717	208	24:02	33:03	08:03	13:02	02:02	04:01	Stable disease
3	F	49	Head	18	Liver, peritonitis, lymph nodes	Ⅳ	7	133	26	02:01	24:02	04:05	09:01	02:02	05:01	Progressive disease
4	M	35	Body	25	Liver, lymph nodes	Ⅳ	6	283	147	02:01	-	09:01	15:01	02:01	05:01	Stable disease
5	F	72	Body	22	Peritonitis, lymph nodes	Ⅳ	14	215	109	02:06	24:02	08:02	12:01	02:01	05:01	Stable disease
6	F	69	Body-tail	45	Lymph nodes	Ⅳ	71+	1050+	545	24:02	33:03	13:02	15:01	04:01	13:01	Stable disease
7	M	39	Head-body	30	Peritonitis	Ⅳ	20	325	290	02:10	24:02	15:01	15:02	02:02	09:01	Stable disease

Stable disease conformation is determined at least for more than 2 mo. Ope: Operation; Cx: Chemotherapy; OS: Overall survival; PFS: Progression-free survival; HLA: Human leukocyte antigen.

Enzyme-linked immunosorbent assay

Throughout the vaccination period, plasma was collected and immediately frozen at -140 ℃ until further use. To investigate the quantitative relationship between IL-6 or IL-8 plasma levels and WT1-specific DTH reactions during vaccinations, the stored plasma was tested for IL-6 or IL-8 every 5 vaccinations using Enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems) according to the manufacturer's instructions. Background cytokine levels were subtracted from each sample.

Statistical analysis

Statistical analyses of prognostic factors for OS and PFS were performed according to the Kaplan-Meier method and evaluated using the log-rank test. IL-6 or IL-8 levels were evaluated in a *t*-test analysis. A *P* value of < 0.05 was considered statistically significant.

RESULTS

Clinical characteristics

All 7 patients with PDA received gemcitabine followed by a combination of gemcitabine and biweekly vaccinations with DC/WT1-Ⅰ/Ⅱ (Figure 1). The clinical characteristics of all PDA patients are presented in Table 1. All patients had disease stage Ⅳ and HLA types of A (A*02:01, A*02:06, or A*24:02), DR (DRB1*04:05, DRB1*08:03, DRB1*15:01, or DRB1*15:02) or DP (DPB1*05:01, or DPB1*09:01). Treatment with the DC/WT1-Ⅰ/Ⅱ vaccine was non-toxic and safe[10]. None of the 7 PDA patients achieved complete or partial response, and 6 (85.7%) exhibited SD. As OS of ≥ 1 year in advanced PDA patients generally indicates that treatment has been beneficial[13], the treated PDA patients were classified into 2 groups: OS ≥ 1 and < 1 year. Three of 7 patients (No. 1, 2, and 6) exhibited OS of ≥ 1 year, and the remaining 4 patients (No. 3, 4, 5, 7) exhibited OS of < 1 year (Table 1). These 3 patients (No. 1, 2, and 6) had long-term SD, resulting in long-term survival (OS ≥ 1 year). From the beginning of treatment, one patient (No. 6) received biweekly 1000 mg/m^2 gemcitabine combined with DC/WT1-Ⅰ/Ⅱ vaccination because of neutropenia. Despite receiving insufficient doses of gemcitabine, the local pancreatic lesions in the patient were stable for more than 1 year (Figure 2A-C, left panel); however, we identified liver metastases at 545 d after the first treatment (Figure 2C, right panel). Therefore, the patient continued treatment with S-1, an oral fluoropyrimidine, or gemcitabine/nab-paclitaxel combined with the DC/WT1-Ⅰ/Ⅱ vaccine. At 545 d after the first treatment, the patient maintained stable primary pancreatic cancer (Figure 2D, left panel) with slightly enlarged liver metastases (Figure 2D, right panel), and survived for more than 1000 d with 100% Karnofsky Performance Status (KPS).

WT1-specific DTH reactions

WT1-specific DTH reactions were not detected prior to treatment in all patients. Chemoimmunotherapy induced strong WT1-specific DTH reactions in all 3 patients (No. 1, 2, and 6) with long-term OS (≥ 1 year) after receiving only one dose of the DC/WT1-Ⅰ/Ⅱ vaccine (Table 2). Moreover, the strong WT1-specific DTH reactions in the 3 super-responders were efficiently maintained during the entire treatment period with at least 30 DC/WT1-Ⅰ/Ⅱ vaccinations (Table 2). However, the DTH reactions in 2 (No. 1 and 2) of the super-responders were severely decreased and became negative at the terminal stage of cancer (35 and 45 vaccinations, respectively). Interestingly, one super-responder (No. 6) remained alive more than 1000 d and received more than 71 vaccinations, resulting in the induction of strong WT1-specific DTH reactions throughout the vaccination period (Table 2). Moreover, a clinical response in terms of SD was achieved in all 3 patients (No. 1, 2, and 6) with strong WT1-specific DTH responses. These patients also maintained 100% KPS during treatment. By contrast,

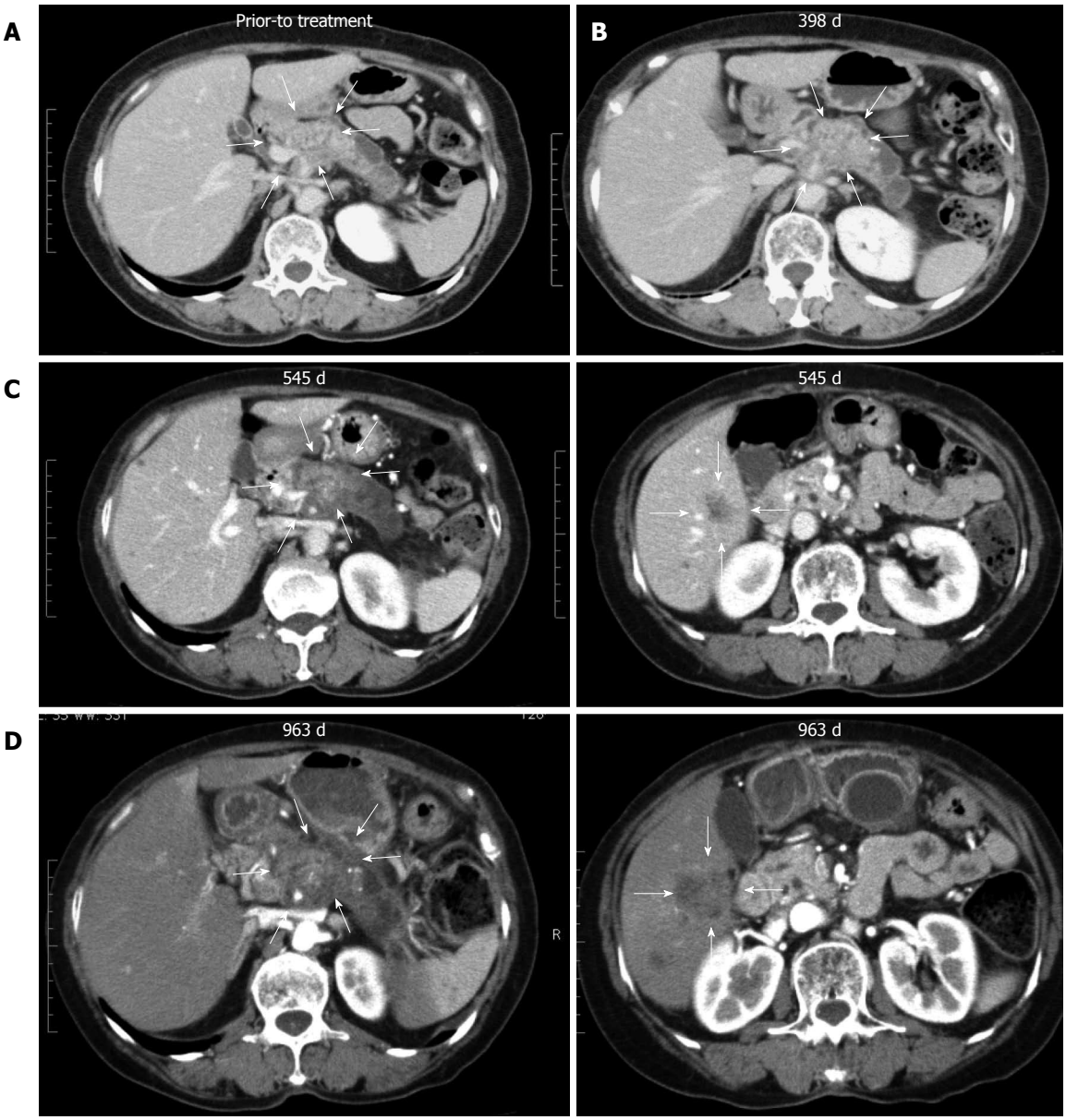

Figure 2 Response assessment in a super-responder, case 6. A: Computed tomography (CT) imaging of case 6 revealing pancreatic body cancer before treatment and no liver metastases; B: CT imaging after chemoimmunotherapy revealed that local pancreatic lesions were stable; C: Local pancreatic lesions were stable (left panel), multiple liver metastases appeared at 545 d after the first treatment (right panel); D: Local pancreatic lesions were stable (left panel), the sizes of multiple liver metastases were markedly increased at 963 d after the first treatment (right panel).

strong WT1-specific DTH reactions were not observed in all 4 nonsuper-responders with OS < 1 year (No. 3, 4, 5, and 7). In the 4 nonsuper-responders, one patient (No. 7) exhibited stable disease and weakly positive DTH reactions against WT1 peptides after 10 to 15 vaccinations; however, the DTH reactions became negative after 20 vaccinations, and the patient died at 325 d after the first treatment. Importantly, all 4 PDA patients with WT1-specific DTH reactions (No. 1, 2, 6, and 7) displayed significantly improved OS compared with the negative control patients (No. 3, 4, and 5) (P = 0.018) (Figure 3A). In particular, all 3 PDA patients with strong DTH reactions (No. 1, 2, and 6) survived more than 1 year, with significantly longer OS compared to the negative control patients (No. 1, 2, and 6) (P = 0.039) (Figure 3B). In addition, the WT1-specific DTH reactions observed in this clinical trial were HLA restricted (Table 2).

Plasma IL-6 level changes in PDA patients after DC/WT1-Ⅰ/Ⅱ vaccination

To assess the prognostic significance of plasma IL-6 levels in PDA patients receiving chemoimmunotherapy using DC/WT1-Ⅰ/Ⅱ vaccines, we analyzed plasma IL-6 levels by quantitative ELISA during treatment (Figure 4A). Prior to treatment, there was no difference in plasma IL-6 levels between super-responders (OS ⩾ 1 year) and nonsuper-responders (OS < 1 year) (Figure 4B). In one nonsuper-responder (No. 5), plasma IL-6 levels were extremely high prior to treatment and

Table 2 Wilms' tumor 1-specific delayed-type hypersensitivity reactions

Patient No.	Gemcitabine		HLA-A*02:01 Vaccine times											HLA-A*24:02 Vaccine times											HLA-DRB1/DPB1 Vaccine times										
	Pre	Post	1	5	10	15	20	25	30	35	40	45	70	1	5	10	15	20	25	30	35	40	45	70	1	5	10	15	20	25	30	35	40	45	70
1	-	-	+	+	++	++	++	++	+	-		ND		+	+	++	++	++	++	+	-		ND		+	+	++	++	++	++	+	-		ND	
2	-	-	-	-	-	-	-	-	-	-	-	-	ND	+	+	++	++	+	+	+	+	+	-	ND	+	+	++	++	+	+	+	+	+	-	ND
3	-	-	-	-					ND					-	-					ND					-	-					ND				
4	-	-	-	-					ND					-	-					ND					-	-					ND				
5	-	-	-	-	-					ND				-	-	-					ND				-	-	-					ND			
6	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	++	++	++	++	++	++	++	++	++	+	+	++	++	++	++	++	++	++	++	++
7	-	-	-	-	-	-	-				ND			-	-	+	+	-				ND					-	+	+	-				ND		

Delayed-type hypersensitivity (DTH) erythema < 1 mm, +; DTH erythema 2-5 mm, ++; DTH erythema > 5 mm, not done (ND).

continuously increased during treatment (60.25-274.5 pg/mL), and we excluded these data from the assessment of the relationship between plasma IL-6 levels and OS. After 1 course of gemcitabine alone, plasma IL-6 levels decreased in some patients but not significantly compared with prior to treatment (Figure 4A). All 3 super-responders (No. 1, 2, and 6) had long-term SD (440, 208, and 545 d, respectively), and plasma IL-6 levels in these patients were significantly decreased compared with the levels prior to treatment after 5 DC/WT1-Ⅰ/Ⅱ vaccinations. This significant decrease continued for 15 vaccinations (Figure 4C). Plasma IL-6 levels in 2 super-responders (No. 1 and 2) were increased after 35 and 45 vaccinations, respectively. However, 1 super-responder (No. 6) maintained low levels of IL-6 for at least 45 vaccinations, resulting in long-term OS. Because of early disease progression in nonsuper-responders (No. 3, 4, 5, and 7), these patients received at least 6 (7, 6, 14, and 20 times, respectively) DC/WT1-Ⅰ/Ⅱ vaccinations. Therefore, we also compared plasma IL-6 levels in super-responders and nonsuper-responders after 5 DC/WT1-Ⅰ/Ⅱ vaccinations. Compared with nonsuper-responders, plasma IL-6 levels in super-responders were significantly lower after 5 vaccinations (Figure 4D), indicating that plasma IL-6 levels decreased in super-responders after the initial vaccinations. In addition, IL-6 levels were remarkably increased at the terminal stage of cancer. Moreover, we assessed the association of IL-6 levels and OS and PFS after 5 DC/WT1-Ⅰ/Ⅱ vaccinations. The PDA patients with low levels of plasma IL-6 (< 2 pg/mL) after 5 vaccinations displayed significantly improved OS (P = 0.025) but not PFS (P = 0.110) compared with those patients with plasma IL-6 ⩾ 2 pg/mL (Figure 5A and B).

Changes in IL-8 plasma levels in PDA patients after DC/WT1-Ⅰ/Ⅱ vaccination

Plasma IL-8 levels in PDA patients after chemoimmunotherapy using DC/WT1-Ⅰ/Ⅱ vaccines were also analyzed by quantitative ELISA (Figure 6A). Prior to treatment, there was no difference in plasma IL-8 levels between super-responders (No. 1, 2, and 6) and nonsuper-responders (No. 3, 4, 5, and 7) (Figure 6B). Importantly, plasma IL-8 levels in all 3 super-responders with long-term SD were significantly decreased after 15 vaccinations compared with the levels observed prior to treatment, and this decrease continued after 25 vaccinations (Figure 6C). However, plasma IL-8 levels in 2 super-responders (No. 1 and 2) were increased after 35 vaccinations. The patients died 582 and 717 d, respectively (Table 1). However, one super-responder (No. 6) maintained low levels of IL-8 until at least 45 vaccinations and survived more than 1000 d. All 3 patients (No. 3, 4, and 5) with negative-DTH reactions against WT1 peptides received less than 15 DC/WT1-Ⅰ/Ⅱ vaccinations (7, 6, and 14 vaccinations, respectively) due to disease progression. Therefore, we also compared plasma IL-8 levels in super-responders at various vaccination periods and nonsuper-responders after 5 DC/WT1-Ⅰ/Ⅱ vaccinations. As shown in Figure 6D, plasma IL-8 levels were significantly higher in nonsuper-responders after 5 DC/WT1-Ⅰ/Ⅱ vaccinations than in super-responders after 15, 20 and 25 vaccinations. In addition, plasma IL-8 levels were remarkably increased at the terminal stage of cancer.

DISCUSSION

In the present study, we analyzed plasma IL-6 and IL-8 levels and assessed WT1-specific DTH reactions in PDA patients receiving DC/WT1-Ⅰ/Ⅱ vaccines. The data

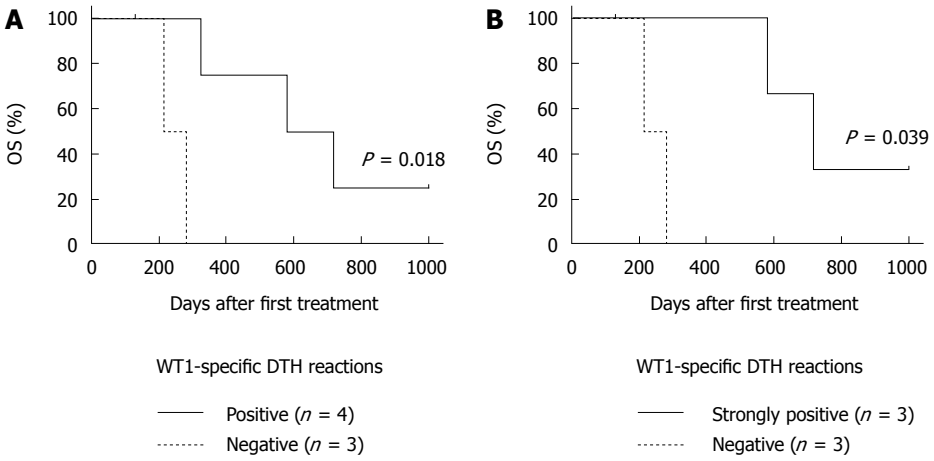

Figure 3 Association of Wilms' tumor 1-specific delayed-type hypersensitivity reactions with overall survival. Kaplan-Meier estimates of overall survival (OS) for patients with PDA treated with dendritic cells (DCs) pulsed with Wilms' tumor 1 (WT1) MHC class Ⅰ and -Ⅱ peptides (DC/WT1- Ⅰ/Ⅱ) combined with chemotherapy. A: OS in WT1-specific delayed type hypersensitivity (DTH)-positive (n = 4) or -negative (n = 3) responders; B: OS in strongly WT1-specific DTH-positive (n = 3) or -negative (n = 3) responders.

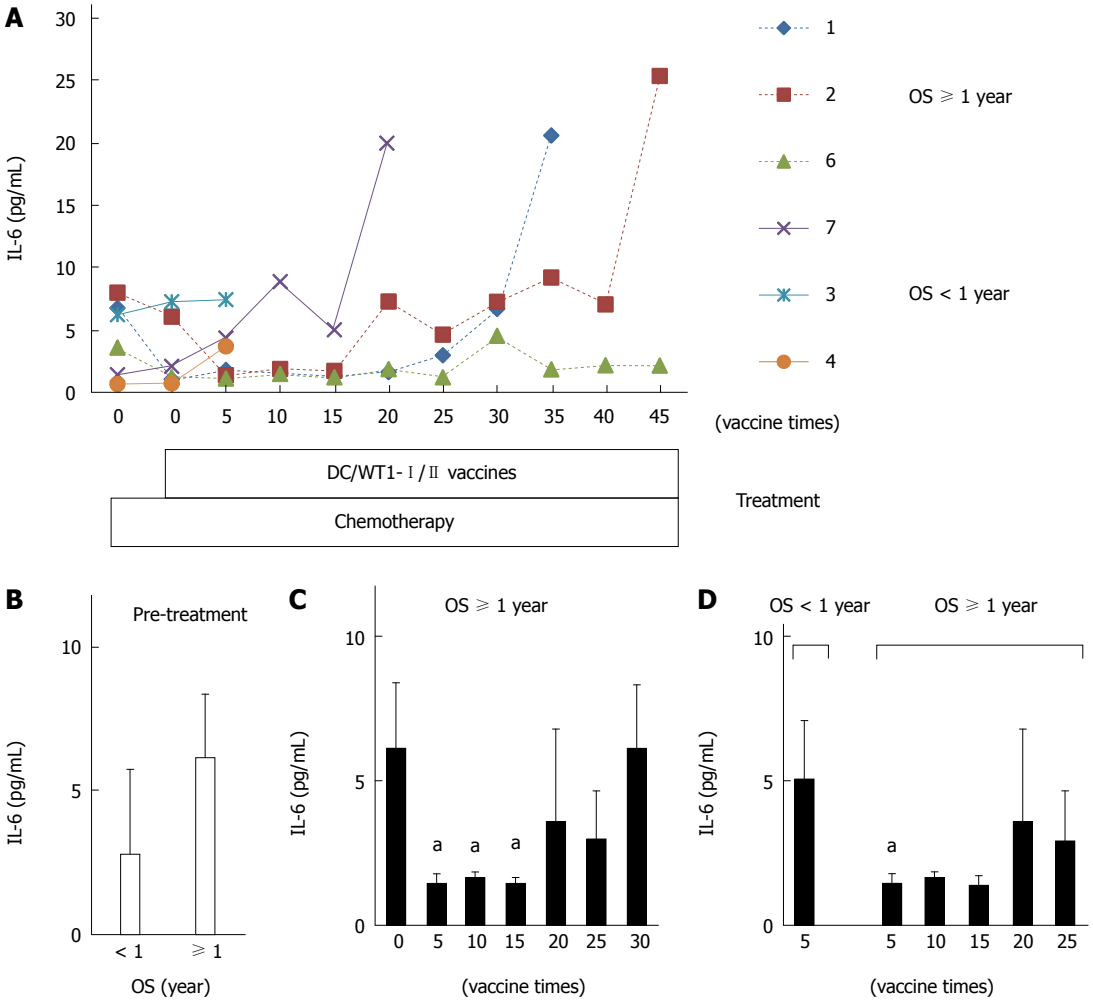

Figure 4 Plasma interleukin-6 levels in pancreatic ductal adenocarcinoma patients receiving chemoimmunotherapy. A: Plasma interleukin (IL)-6 levels in 6 patients during treatment; B: Comparison of plasma IL-6 levels prior to treatment between super-responders [overall survival (OS) ⩾ 1 year] and nonsuper-responders (OS < 1 year); C: Comparison of plasma IL-6 levels in super-responders prior to treatment and post-vaccination; D: Comparison of plasma IL-6 levels after 5 vaccinations between nonsuper-responders and super-responders after 5, 10, 15, 20, and 25 vaccinations. The values are expressed as mean ± SD. $^{a}P < 0.05$.

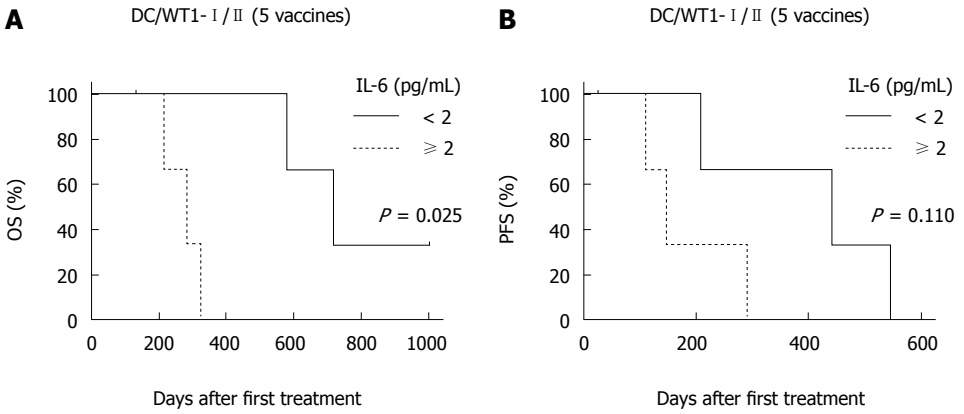

Figure 5 Association of plasma interleukin-6 levels with overall survival and progression-free survival. The plasma interleukin (IL)-6 levels of 7 pancreatic ductal adenocarcinoma patients receiving chemoimmunotherapy were analyzed after 5 vaccinations. Kaplan-Meier estimates of OS (A) and PFS (B) in these patients.

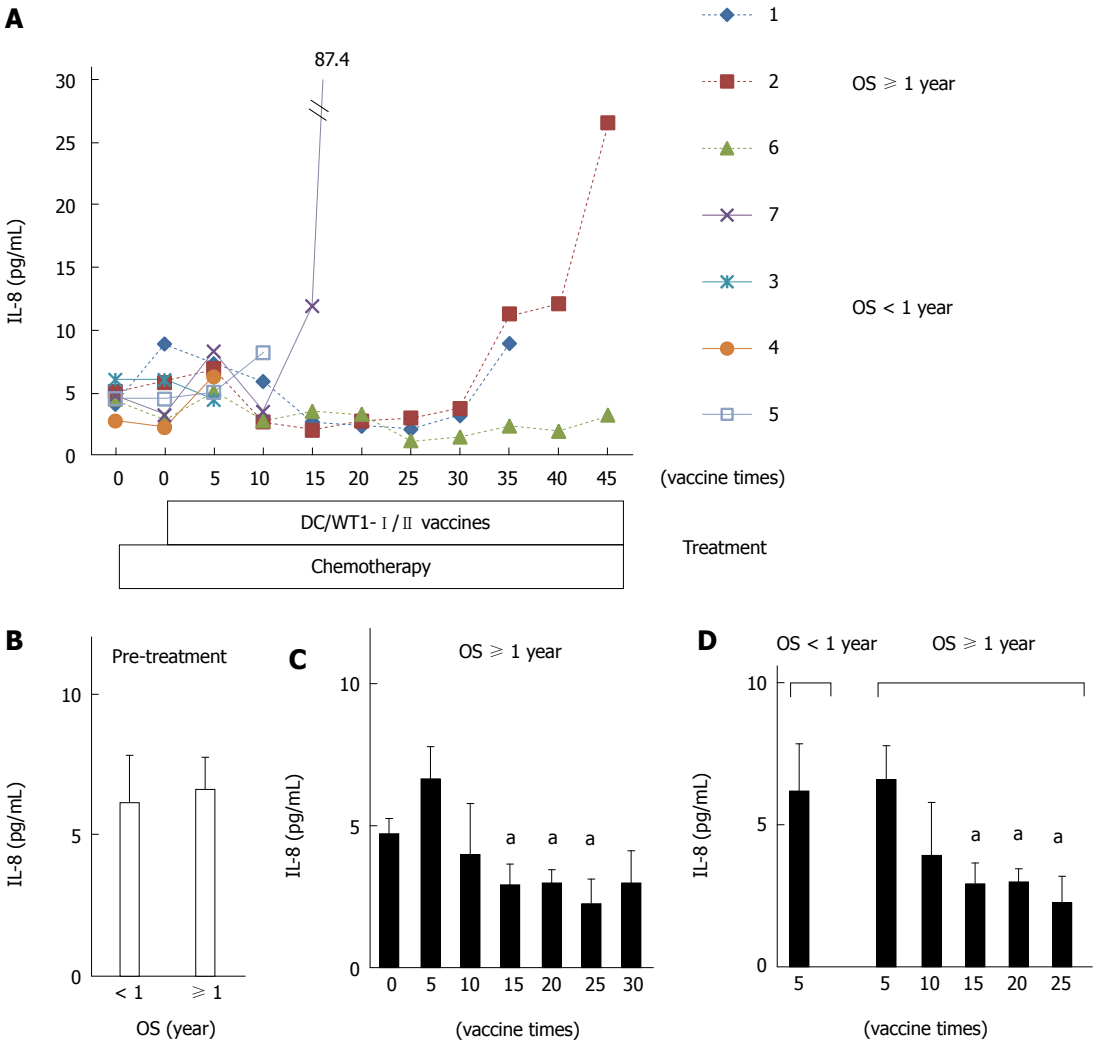

Figure 6 Plasma interleukin-8 levels in pancreatic ductal adenocarcinoma patients receiving chemoimmunotherapy. A: Plasma interleukin (IL)-8 levels in 7 patients during treatment; B: Comparison of plasma IL-8 levels prior to treatment between super-responders [overall survival (OS) ⩾ 1 year] and nonsuper-responders (OS < 1 year); C: Comparison of plasma IL-8 levels in super-responders prior to treatment and post-vaccination; D: Comparison of plasma IL-8 levels after 5 vaccinations in nonsuper-responders and super-responders after 5, 10, 15, 20, and 25 vaccinations. The values are expressed as means ± SD. $^{a}P < 0.05$.

presented herein suggest that long-term decreases in plasma IL-6 and IL-8 levels compared to the early treatment period are associated with the induction of WT1-specific DTH reactions. Significantly prolonged OS was observed in PAD patients with strong WT1-specific DTH reactions. Thus, long-term low levels of plasma IL-6 and IL-8 during chemoimmunotherapy may be prognostic markers of clinical outcomes.

In a recent clinical trial, to simultaneously activate WT1-specific $CD4^+$ and $CD8^+$ T cells, mature DCs were pulsed with a mixture of three types of WT1 peptides, including MHC class Ⅰ and Ⅱ-restricted epitopes, and injected into one site biweekly as a cancer vaccine[10]. DC/WT1-Ⅰ/Ⅱ vaccinations not only induced WT1-specific $CD4^+$ and CD8 T cells but also maintained long-term WT1-specific memory $CD8^+$ T cells[10]. The phase 1 study indicated that WT1-specific DTH reactions induced after treatment with DC/WT1-Ⅰ/Ⅱ combined with chemotherapy were associated with long-term disease stability in advanced PDA. Moreover, the WT1-specific CTL responses continued throughout long-term vaccination and were associated with long-term OS[10]. With chemotherapy alone, SD is often transient and not considered indicative of true antitumor activity. Because, cancer vaccines are not as rapidly effective as cytotoxic agents, SD is considered an indicator of a meaningful therapeutic effect[14]. Long-term SD in patients receiving immunotherapy may be unique to cancer vaccines and is considered evidence of activity[10]. In the present study, we assessed immunological responses in 7 patients with stage Ⅳ PDA who received DC/WT1-Ⅰ/Ⅱ combined with chemotherapy. Three patients displayed strong WT1-specific DTH reactions in long-term chemoimmunotherapy, resulting in long-term OS ⩾ 1 year. Antigenic peptide-specific DTH is an inflammatory reaction that is primarily mediated through $CD4^+$ effector-memory T cells primed by the cancer vaccines[15]. A significant correlation between favorable clinical outcomes and the induction of a cancer vaccine-related antigen-specific DTH reaction has been observed[15]. Therefore, the long-term maintenance of strong WT1-specific DTH reactions in these 3 patients might be associated with long-term SD, resulting in prolonged survival. Indeed, one patient had long-term SD and continuously strong WT1-specific DTH, surviving at least 1000 d with 100% KPS.

The plasma levels of circulating IL-6/-8 were also analyzed by ELISA, a more quantitative analysis than the multiplexed measurement system of flow cytometry, and the cytokines levels were compared with the clinical outcomes. In the present study, none of the 7 PDA patients achieved complete or partial response, and 6 (85.7%) exhibited SD. Three patients (No. 1, 2, and 6) exhibited long-term SD, resulting in long-term survival. These 3 patients displayed significantly decreased levels of plasma IL-6 after 5 to 15 vaccinations. IL-6 is secreted from a variety of cells, primarily lymphocytes, macrophages, monocytes, fibroblasts, endothelial cells, and keratinocytes[16]. Some tumor cells also secrete IL-6[16]. One histological hallmark of PDA is the presence of a highly desmoplastic stroma, including several inflammatory cell populations, such as fibroblast, stellate, endothelial, endocrine, and immune cells, all of which produce different inflammatory cytokines[17]. The multifunctional inflammatory cytokine IL-6 plays a role in the development and progression of PDA by directly affecting tumorigenesis[18]. Moreover, IL-6 regulates the secretion of vascular endothelial growth factor in PDA cells, thereby stimulating angiogenesis and tumor vascularization resulting in lymphatic and distant metastasis and disease progression[18-20]. Therefore, the maintenance of low plasma IL-6 levels in 3 patients receiving chemoimmunotherapy using DC/WT-1-I/Ⅱ at early vaccination periods (5 vaccines) and its continuation for 15 vaccinations may be responsible for the prolonged survival of the PDA patients. Importantly, these patients also maintained long-term strong WT1-specific DTH reactions. By contrast, extremely high levels of IL-6 and IFN-γ (data not shown) were detected in a nonsuper-responder (No. 5). The expression of IL-6 is potentiated by IFN-γ *via* prolonged NF-κB activation[21]. Three super-responders exhibited slightly increased IFN-γ production from $CD4^+$ and $CD8^+$ T cells upon stimulation with WT1 peptides *in vitro*[10]. The low levels of plasma IL-6 in the super-responders patients did not interfere with the induction of WT1-specific antitumor immune responses and were associated with a prolonged survival period.

After chemoimmunotherapy, significantly decreased plasma IL-8 levels were also obvious in all 3 super-responders with strong WT1-specific DTH reactions. Compared with IL-6, significantly decreased levels of plasma IL-8 were observed in the patients after longer periods of treatment (from 15 to 25 vaccinations). Although tumor-associated macrophages and monocytes are the most likely source of IL-8, this cytokine is also overexpressed in most human PDA cells under inflammatory conditions[22]. Several cell types within the tumor microenvironment produce a variety of cytokines. Differences in cells producing inflammatory cytokines such as IL-6 and IL-8 may underlie periods of decreased plasma IL-6 and IL-8 levels. In the tumor environment, these cytokines interact with other cell types in a complex network. Importantly, IL-8 plays a major role in the progression of PDA by promoting proliferation, migration, angiogenesis and metastasis[23]. Elevated levels of circulating IL-8 are associated with poor clinical outcome in patients with PDA and have been suggested as a prognosis marker[24,25]. Therefore, a significant decrease in plasma IL-8 levels might also be associated with good prognosis in PDA patients receiving the chemoimmunotherapy using DC/WT1-Ⅰ/Ⅱ. Indeed, all 3 super-responders with strong WT1- DTH reactions maintained low levels of IL-8 after 25 vaccinations and exhibited prolonged SD. However, plasma IL-8 and

IL-6 levels in 2 super-responders (No. 1 and 2) were increased at the terminal stage of cancer, and these patients had WT1-negative DTH reactions. However, one super-responder (No. 6) maintained low levels of IL-8 and IL-6 and displayed strong WT1-specific DTH reactions after at least 45 vaccinations. Recently, we reported that IL-8 secretion from tumor cells enhances the generation and activation of CD163-positive M2 macrophages producing IL-10, leading to poor clinical outcomes in patients with cancer[26]. That finding is consistent with the results of the present study demonstrating that long-term low levels of circulating IL-8 in PDA patients receiving DC/WT1-Ⅰ/Ⅱ is associated with the maintenance of strong WT1-specific DTH reactions, resulting in good clinical outcomes.

In conclusion, both IL-6 and IL-8 were maintained at low levels in all 3 PDA patients with strong WT1-specific DTH reactions who received DC/WT1-Ⅰ/Ⅱ combined with chemotherapy. All 3 PDA patients exhibited long-term SD and prolonged survival times (582 to more than 1000 d). One patient with long-term strong WT1-specific DTH reactions maintained decreased levels of plasma IL-6 and IL-8 during therapy and maintained long-term SD, resulting in survival for more than 1000 d. Therefore, the maintenance of low plasma IL-6 and IL-8 levels may be associated with immunogenic changes in the desmoplastic stroma. Low levels of plasma IL-6 and IL-8 with strong WT1-DTH reactions may be a prognostic factor for PDA patients following chemoimmunotherapy using DC/WT1-Ⅰ/Ⅱ. These cytokine interactions are associated with tumor growth and progression, invasion and metastasis, angiogenesis and immune evasion. Although targeting IL-6 and IL-8 may improve not only clinical outcome but also the response to treatment in PDA patients, it is not clear if IL-6/-8-signaling inhibitors will translate into clinical benefits for PDA[19]. A limitation of the present study is the relatively small sample size. Further studies are needed to evaluate the clinical significance of circulating IL-6/-8 levels in PDA patients treated with DC/WT1-Ⅰ/Ⅱ combined with chemotherapy.

COMMENTS

Background

$CD4^+$ T cells prime and maintain antigen-specific $CD8^+$ CTLs and play a direct role in the destruction of tumor cells. Therefore, the stimulation of both $CD4^+$ and $CD8^+$ T cells is an efficient strategy for treating patients with pancreatic ductal adenocarcinoma (PDA). The authors had conducted a phase 1 clinical study in PAD patients to examine the clinical and immunological responses to dendritic cells (DCs) pulsed with multiple major histocompatibility complex class Ⅰ and Ⅱ-restricted Wilms' tumor 1 (WT1) epitopes (DC/WT1-Ⅰ/Ⅱ) in combination with chemotherapy. The combination of DC/WT1-Ⅰ/Ⅱ and chemotherapy were associated with disease stability in PAD patients.

Research frontiers

Authors investigate the association of plasma levels of interleukin (IL)-6 and -8 with WT1-specific immune responses and clinical outcomes in patients with PDA treated with DC/WT1-Ⅰ/Ⅱ combined with standard chemotherapy. The authors reported the association of plasma IL-6 and -8 levels and WT1 peptide-specific DTH in PDA patients during long-term chemoimmunotherapy.

Innovations and breakthroughs

Authors showed that plasma IL-6/-8 levels in PDA patients during long-term treatments. Prolonged low levels of plasma IL-6/-8 may be a prognostic marker for the clinical outcomes of chemoimmunotherapy.

Applications

The study's results suggest that plasma IL-6/-8 in PDA patients during chemoimmunotherapy using DC/WT1-Ⅰ/Ⅱ is a prognostic biological marker for assessing the induction of WT1-specific immune responses and long-term overall survival.

Terminology

The WT1 is highly expressed in various types of cancers, including PDA and has been found to be highly immunogenic. Thus, WT1 is one of the excellent tumor-associated antigens for the target of cancer immunotherapy.

Peer-review

The authors reported that prolonged low levels of plasma IL-6/-8 in PDA patients may be a prognostic marker for the clinical outcomes of chemoimmunotherapy. The data are well represented, organized, and clear.

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ORIGINAL ARTICLE

Clinical Trials Study

Effects of daily telephone-based re-education before taking medicine on *Helicobacter pylori* eradication: A prospective single-center study from China

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Author contributions: Lan CH designed the research; Wang CH, Yang J, Li CX and Yang YY performed the research; Han R and Chen DF contributed new reagents or analytic tools; Wang CH and Liao ST analyzed the data; Liao ST and Lan CH wrote the paper.

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Institutional review board statement: The study was reviewed and approved by Daping Hospital, Third Military Medical University Institutional Review Board.

Clinical trial registration statement: This study is registered at http://www.chictr.org.cn/showproj.aspx?proj=4382. The registration identification number is ChiCTR-TRC-14005193.

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

Conflict-of-interest statement: Chun-Hua Wang, Sheng-Tao Liao, Jun Yang, Chun-Xia Li, Ying-Ying Yang, Ran Han, Dong-Feng Chen, and Chun-Hui Lan have no conflicts of interest or financial ties to disclose.

Data sharing statement: No additional data are available.

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Abstract

AIM: To investigate the effects of daily telephone-based re-education (TRE) before taking medicine for the eradication of *Helicobacter pylori* (*H. pylori*) on the compliance and the eradication rate in a Chinese patient population.

METHODS: A prospective, physician-blinded, randomized, controlled clinical study was conducted. The patients were randomly assigned to receive TRE every day before taking medicine (TRE group) or no TRE (control group). The patients in the TRE group received regular instructions before taking medicine for the eradication of *H. pylori* during the entire course

of treatment through telephone calls. The patients in the control group received detailed instructions at the time of seeing a doctor for the guidance. The primary outcome was the *H. pylori* eradication rate after treatment. The secondary outcomes included the clinical remissions after treatment, adverse events, compliance, and patients' satisfaction.

RESULTS: A total of 140 patients were randomized, 70 to the TRE group and 70 to the control group. As the primary outcome, the *H. pylori* eradication rates in the TRE and control groups were 62.7% and 71.2% in per protocol analysis (P = 0.230), and 52.9% and 52.9% in intention-to-treat analysis (P = 0.567), respectively. As the secondary outcomes, there were no significant differences in the patients' satisfaction between the two groups (good, 79.7% *vs* 76.9%; fair, 13.6% *vs* 19.2%; poor, 6.7% *vs* 3.9%, for the TRE group and control group, respectively; $P > 0.05$ for all); the rates of adverse effects were 15.2% and 63.5% in the TRE and control groups, respectively ($P < 0.001$); the compliance rates in the TRE and control groups were 85.7% and 74.3%, respectively (P = 0.069).

CONCLUSION: Daily TRE before taking medicine had no significant impact on the patients' compliance, satisfaction, or *H. pylori* eradication, but reduced the rate of adverse events.

Key words: *Helicobacter pylori*; Eradication; Telephone re-education; Compliance; Adverse events

Core tip: Compliance is an important factor affecting *Helicobacter pylori* (*H. pylori*) eradication. The present study is the first attempt to evaluate the telephone re-education (TRE) in *H. pylori* treatment in China. The daily TRE neither improved the eradication rate nor the patients' compliance or satisfaction, but decreased adverse effects. Meanwhile, adverse effects may not be the main reason for poor compliance. Our results suggest that compliance is not the important reason for a decreased *H. pylori* eradication rate in China.

Wang CH, Liao ST, Yang J, Li CX, Yang YY, Han R, Chen DF, Lan CH. Effects of daily telephone-based re-education before taking medicine on *Helicobacter pylori* eradication: A prospective single-center study from China. *World J Gastroenterol* 2015; 21(39): 11179-11184 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i39/11179.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i39.11179

INTRODUCTION

Helicobacter pylori (H. pylori) is a pathogen that infects more than 50% of the human population, resulting in high healthcare costs worldwide[1]. Although some of the *H. pylori*-positive individuals may remain asymptomatic through their life span, the infection can cause chronic gastritis in almost 100% of infected individuals, even resulting in severe diseases, such as atrophic gastritis, peptic ulcer disease, and gastric cancer[2]. Recently it has been revealed that poor compliance is often seen in patients undergoing treatment for the eradication of *H. pylori*[3]. Poor compliance and bacterial resistance are two major factors that cause the eradication rates of *H. pylori* to decrease to an unacceptable level ($\leqslant$ 80%)[4,5].

Compliance in *H. pylori* treatment has a great influence on treatment failures in antibiotic-sensitive patients and on the development of antibiotic resistance[6]. Multiple factors such as complexity and duration of the treatment can affect the patients' compliance. Several methods had previously been tried to enhance patients' compliance with instructions for *H. pylori* treatment, but the results are inconsistent. Adjuvant treatment (especially with probiotics) can improve the compliance[7].

Taking phone interview on the last day of therapy and returned pill counting can improve patients' compliance, but attempts to increase compliance may have no impact on the treatment outcomes[8]. The doctor-patient relationships, including the relations with pharmacists and nurses, can also play an important role in improving compliance and eradication of *H. pylori*[9]. In addition, structured aftercare and follow-up often help improve the compliance and subsequent *H. pylori* eradication rate[10,11].

China is among the countries with a high prevalence of *H. pylori* infection with a rate of 50%-80%. Several factors may affect the compliance of *H. pylori* treatment in Chinese patients such as lack of detailed guidance on drug administration, lack of detailed instruction or consultation from gastrointestinal doctors due to busy schedule, misunderstanding of or concerns over the side effects of Western medicine, and preference of use of traditional Chinese medicine due to the belief of having fewer adverse effects[12]. Therefore, there is an urgent need for improving the compliance of *H. pylori* treatment in China.

Telephone re-education (TRE) is often used to improve treatment compliance[6], but has not been well studied in China. We hypothesized that compliance of *H. pylori* infected patients and *H. pylori* eradication rate could be improved by TRE every day before taking medicine, reminding the patients of the detailed information related to the time and dose of medicines, answering the patients' questions, and comforting and encouraging them to continue the treatment. The purpose of the present study was to evaluate the effects of the intervention with TRE on the rate of *H. pylori* eradication and on other clinically relevant outcomes, such as the clinical symptoms after treatment and treatment-related adverse effects.

MATERIALS AND METHODS

Patients

The present study employed a prospective, physician-blinded, randomized, controlled single-center study design, and was conducted in consecutive outpatients at the Department of Gastroenterology of Daping Hospital, Chongqing, China, from September 2014 to November 2014. The study protocol and informed consent form were reviewed and approved by the Ethics Review Committee of Daping Hospital, Third Military Medical University, Chongqing, China [Approval No. (2014) 06], and the study was registered with the Chinese Clinical Trial Register (ChiCTR-TRC-14005193). Each of the patients provided written informed consent before enrolment to the trial.

Patients were randomized to either a TRE group or a control group at the time of first clinical visit, by using a computer generated random number kept in a sealed opaque envelope. At least two telephone numbers for each of the patients or their relatives living together were recorded for the TRE. All patients were instructed not to tell physicians and/or investigators about their preparation method and when they received instructions in any time of the study (before, during and after the procedure)

The inclusion criteria were as follows: (1) outpatients aged 18-60 years with chronic gastritis or gastroduodenal ulcer; (2) confirmed diagnosis of *H. pylori* infection by at least one of the following methods: ^{13}C-urea breath test, histology, rapid urease test or bacterial culture; (3) an indication of *H. pylori* eradication treatment; and (4) ability and willingness to participate in the study.

The exclusion criteria were as follows: (1) advanced chronic disease that would not allow the patient to complete follow-up or attend visits; (2) allergy to any of the drugs used in this study; (3) previous gastric surgery; (4) pregnancy or breastfeeding (female participants with childbearing potential were required to use medically accepted contraception for the duration of the study); (5) alcohol or drug abuse; (6) previous *H. pylori* eradication treatment; and (7) taking antibiotics or bismuth salts within two weeks before the study.

Intervention

The patients in the TRE group received detailed instructions at the time of seeing a doctor for the guidance of clinical medication and reexamination at four weeks after the treatment. The detailed instructions of rational drug use and periodic review were given by another doctor through telephone call every day before taking medicine. The 10-d treatment was the triple therapy, including esomeprazole (AstraZeneca Pharmaceutical Co, London, United Kingdom; Lot H20046379; 20 mg/12 h), amoxicillin (Zhuhai Union Pharmaceutical Co. ltd., Zhongshan, China, H44021351; 1 g/12 h), and clarithromycin (Shanghai Abbott Laboratories Co. Ltd., Shanghai, China, J20050067; 500 mg/12 h). Symptom relieving drugs including gastric mucosal protective drugs, cardiovascular drugs, and other medications were allowed to be used in both groups when needed. At four weeks after treatment, the patients received telephone calls to schedule reexamination through ^{13}C-urea breath test to confirm eradication.

The patients in the control group received detailed instructions at the time of seeing a doctor for the guidance of clinical medication and reexamination at four weeks after treatment. The treatment plan was identical as that in the TRE group.

Trial outcomes

The primary outcome was the eradication of *H. pylori* at four weeks after treatment. The eradication of *H. pylori* was confirmed by the ^{13}C-urea breath test. The secondary outcomes included the compliance, clinical symptom remission after treatment, adverse events, and patients' satisfaction. The satisfaction of the patients were evaluated and recorded using a ten-point scale (poor, 1-4; fair, 5-7; good, 8-10) based on questionnaire in a physician-blinded fashion.

Calculation of sample size

The sample size calculation was performed by assuming a 25% difference in the rate of *H. pylori* eradication after treatment. The rate of standard triple therapy in China was about 75%. We calculated that at least 116 patients were needed for the study by using a significance level (α) of 0.05 and a power of 80% with a two-tailed test. However, from our previous experience, approximately 20% of patients may withdraw from the trial or be lost to follow-up. We estimated that a total of 140 patients would be sufficient to detect a significant difference in the primary outcome.

Statistical analysis

The intention-to-treat (ITT) and per-protocol (PP) analyses were used to assess the primary outcome. Categorical variables were analyzed using χ^2 tests or Fisher's exact test, as appropriate. Continuous variables were expressed as mean ± SD and analyzed using Student's *t*-test. Analyses were performed with SPSS software V.19.0 for Windows. A P value < 0.05 was considered statistically significant.

RESULTS

Patients' characteristics

We screened 286 outpatients with *H. pylori* infection; 146 of them were excluded (82 met exclusion criteria and 64 were unwilling to participate in the study); 140 were randomized to the TRE group (n = 70) or the control group (n = 70). Eleven subjects in the TRE group and 18 in the control group withdrew from the

Table 1 Baseline characteristics of the patients included in this study *n* (%)

Characteristic	TRE group (*n* = 70)	Control group (*n* = 70)	*P* value
Sex (M/F)	25/45	28/42	1.000
Age (yr)	42.9 ± 10.7	45.9 ± 9.2	0.098
BMI (kg/m^2)	23.1 ± 4.0	23.7 ± 3.7	0.677
Grade of education			0.199
Elementary school or no education	5 (7.1)	9 (12.9)	
Higher than elementary school	65 (92.9)	61 (87.1)	
Residence			0.604
Country	8 (11.4)	8 (11.4)	
City	62 (88.6)	62 (88.6)	
Digestive tract hemorrhage	5 (7.1)	3 (4.3)	0.529
Family gastric cancer history	4 (5.7)	2 (2.9)	0.340
Endoscopy results			
Chronic gastritis	55 (71.2)	59 (84.3)	0.257
Peptic ulcer	14 (20.0)	10 (14.3)	0.251
Helicobacter pylori infection	70 (100.0)	70 (100.0)	

Values are expressed as mean ± SD, % or *n* (%). BMI: Body mass index; TRE: Telephone re-education; M: Male; F: Female.

Table 2 Effects of telephone re-education on the symptoms after treatment of *Helicobacter pylori* eradication *n* (%)

Symptom	TRE group (*n* = 59)	Control group (*n* = 52)	*P* value
Pain	14 (23.7)	10 (19.2)	0.367
Burning sensation	3 (5.1)	5 (9.6)	0.290
Acid reflux	4 (6.8)	3 (5.8)	0.571
Nausea and vomiting	3 (5.1)	1 (1.9)	0.358
Belching	2 (3.4)	3 (5.8)	0.440
Abdominal distension	8 (13.6)	2 (3.8)	0.071
Poor appetite	1 (1.7)	1 (1.9)	0.720

TRE: Telephone-based re-education.

study or were lost to follow-up for various reasons including a busy schedule, remission of symptoms and stopping medication in treatment process, unsatisfying efficacy, giving up treatment, adverse events, and others. There was no significant difference in the number of patients lost in the follow-up between the two groups (11 *vs* 18, *P* = 0.069). Finally, 59 in the TRE group and 52 in the control group completed the treatment for the eradication of *H. pylori*.

The baseline characteristics of the patients in both groups were well balanced (Table 1), with no significant differences between the two groups.

Outcomes of treatment

In the PP analysis of the primary outcome, the *H. pylori* eradication rates of the TRE and control groups were 62.7% (37/59) and 71.2% (37/52) (*P* = 0.230), while in the ITT analysis, the rates were 52.9% (37/70) and 52.9% (37/70) (*P* = 0.567), respectively.

There were no significant differences in the symptoms after treatment between the two groups (*P* > 0.05 for all; Table 2). The rate of adverse effects in the TRE group was significantly lower than that of the control group (*P* < 0.001; Table 3). The taste disorder was significantly lower in the TRE *vs* the control group (6.8% *vs* 53.8%, *P* < 0.001)

Table 3 Effects of telephone re-education on the adverse events after *Helicobacter pylori* treatment *n* (%)

Adverse event	TRE group (*n* = 59)	Control group (*n* = 52)	*P* value
Skin rash	2 (3.4)	0 (0.0)	NS
Headache	1 (1.7)	0 (0.0)	NS
Sore throat	0 (0.0)	2 (3.8)	NS
Taste disorder	4 (6.8)	28 (53.8)	< 0.001
Diarrhea	2 (3.4)	3 (5.8)	0.44
Total	9 (15.2)	33 (63.5)	< 0.001

NS: Not significant; TRE: Telephone-based re-education.

Table 4 Satisfaction of patients included in this study *n* (%)

	TRE group	Control group	*P* value
Satisfaction			
Good	47 (79.7)	40 (76.9)	0.452
Fair	8 (13.6)	10 (19.2)	0.290
Poor	4 (6.7)	2 (3.9)	0.401

TRE: Telephone-based re-education.

The compliance rate in the TRE group (84.3%) was slightly higher; however non-significant than that of the control group (74.3%, *P* = 0.069). The results of patients' satisfaction are shown in Table 4. There was no significant difference in patients' satisfaction between the two groups (*P* > 0.05).

DISCUSSION

The triple therapy as a traditional standard care is widely used in China, but the eradication rate is decreasing due to various reasons such as drug resistance, poor compliance, high bacterial loads, and genic polymorphisms of cytochrome P450 proteins 2C19 (CYP2C19)[2,13-15]. Therefore, the standard triple therapy does not reach the acceptable threshold of 80% eradication rate in most contexts[5,16]. Most studies have focused on the antibiotic resistance. In fact, improving compliance is relatively simple and less costly compared with other measures to improve the therapeutic outcome. The improvement in compliance can be accomplished through education. In the present study, the *H. pylori* eradication rates in the TRE and control groups were 62.7% and 71.2% (PP), and 52.9% and 52.9% (ITT), respectively.

Failure to comply with the anti-*H. pylori* therapy (AHT) requirements by the patient often results in treatment failure[6,17]. In a previous study, patients with good compliance had a higher AHT effectiveness rate (96%) than those with low compliance (69%)[17]. The major reason for poor compliance was the development of an adverse event during the course

of AHT[18], and the adverse events were found to be different with different AHT regimens[19]. In our study, the rates of adverse effects were 15.2% and 63.5% in the TRE and control groups, respectively. The reasons for the difference may be a result of telephone follow-up every day, including timely resolve, comfort and relief of the symptoms among the TRE patients. The studies with a seven-day AHT regimen have shown a 41% frequency of adverse effects, which provoked cessation of therapy in 3%-10% of the patients[20]. If the treatment time is prolonged to 10-14 d, the development of adverse effects can be seen in more than half of the patients[21].

In order to improve the compliance of *H. pylori* eradication, detailed instructions of rational drug use and periodic review were provided by telephone call every day before taking medicine. A follow-up telephone call after initiation of therapy in a previous study suggested that although adverse effects were common between enhanced compliance program and control groups, most patients were able to complete 60% or more of the two-week regimen[20]. There was no statistically significant difference between the two test groups in the number of patients taking more than 60% of the medications, and the number of patients taking more than 90% of the medications; an enhanced compliance program further improved the percentage of medications taken[22].

However, similar studies found that the enhanced compliance had no impact on the treatment outcome and adverse effects were very common[8].

In the present study, TRE every day before taking medicine could not significantly improve the compliance or the *H. pylori* eradication rate. No statistically significant differences were found between TRE and control groups regarding the symptoms after treatment; however, the adverse effects in the TRE were significantly fewer than those in the control group. The results indicated that adverse effects might not be the major reason for poor compliance and less effectiveness in the eradication of *H. pylori*.

Although adverse effects are common in standard therapies, they are rarely severe. In our study, the adverse effects included taste disorder, diarrhea, skin rash, headache and sore throat. The most common adverse events reported in most studies are diarrhea, nausea, and vomiting. Using the standard first-line triple therapy, a multi-center study has found that the overall rate of adverse events was 53.3%[23]. The evidence supporting concurrent administration of probiotics to lessen the side-effects of triple therapy is still equivocal. While Kim *et al*[24] found that probiotics had no effect on the side-effect profile, but increased the rates of eradication, another study revealed that probiotics reduced side-effects and did not affect the eradication rate[25]. In our study, the adverse effects in the TRE group were significantly less than that of the control group, but the *H. pylori* eradication rates were similar between the two groups. Although the compliance increased in the TRE group, it was non-significant compared to the control group. Moreover, there were no differences in the satisfaction of patients between the two groups. It seems that the patients neither recognized the difference nor acknowledged the effects of the TRE. It is possible that daily telephone calling was exaggerated and perhaps made the patients feel uncomfortable. The effectiveness and acceptance of TRE may be dependent on the type of disease and the intervention. For instance, in a study conducted in China, it has been reported that TRE one day prior to colonoscopy improved the compliance, quality of bowel preparation, and the polyp detection rate[26]. Our results suggested that TRE may not be the best way to improve compliance in *H. pylori* treatment and that modifications of the TRE intervention may be required or other better approaches are needed.

In conclusion, the present study represented the first attempt to evaluate the TRE in *H. pylori* treatment in China. Although the daily TRE did not improve the *H. pylori* eradication rate, compliance, or patients' satisfaction, it decreased adverse effects. Meanwhile, adverse effects may not be the main reason for poor compliance. Our results may help develop an effective plan for improving compliance and therapeutic outcome of *H. pylori* therapy.

COMMENTS

Background

Compliance with therapy is one of the most important factors in *Helicobacter pylori* (*H. pylori*) eradication. The effects of telephone re-education (TRE) daily before taking medicine for *H. pylori* treatment on the compliance and the *H. pylori* eradication rate have not yet been studied in China.

Research frontiers

Most studies have mainly focused on the antibiotic resistance in the treatment of *H. pylori* eradication. Improving compliance is a relatively simple and less costly approach to improving the therapeutic outcome, compared with other measures. The improvement in compliance can be accomplished through effective patient education program.

Innovations and breakthroughs

In the present study, an innovative TRE program was developed and evaluated in patients undergoing *H. pylori* treatment in China. The most important findings were that daily TRE before taking medicine reduced the rate of adverse events, but had no significant impact on the patients' compliance, satisfaction, or *H. pylori* eradication rate.

Applications

The results from the present study suggest that compliance is not the main reason for a decreased *H. pylori* eradication rate in China. Additionally, adverse effects may not be the main reason for poor compliance. These results would help develop a more effective plan for improving compliance and therapeutic outcome of *H. pylori* therapy in China.

Terminology

Daily TRE: The detailed instructions of rational drug use and periodic review were given to the patient by physicians through telephone call every day before taking medicine.

Peer-review

The authors conducted a new study to investigate the effects of daily TRE program before taking medicine on the compliance and the eradication rate

in patients with the treatment for eradication of *H. pylori*. It is believed that this is the first study of this kind in China. The authors provided sufficient data to support their conclusion that daily TRE before taking medicine had no significant impact on the patients' compliance, satisfaction, or *H. pylori* eradication, but reduced the rate of adverse events. The research is interesting and the study design and the results are clearly presented. Future large-scale studies should be conducted to develop an effective way to improve the outcome of *H. pylori* eradication therapy in China and other countries as well.

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SYSTEMATIC REVIEWS

Managements of recurrent hepatocellular carcinoma after liver transplantation: A systematic review

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Author contributions: de'Angelis N and Azoulay D were at the origin of the work; de'Angelis N and Carra MC performed the systematic review of the literature (search, evaluation, selection, and quality assessment of the articles), data extraction, data analysis, and manuscript drafting; Landi F was the third blind reviewer and participated at the manuscript drafting and corrections; Azoulay D contributed at the final version of the manuscript with corrections and criticism.

Conflict-of-interest statement: Nicola de'Angelis, Filippo Landi, Maria Clotilde Carra and Daniel Azoulay have no conflict of interest to disclose in relation to the present systematic review.

Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at nic.deangelis@yahoo.it. No additional data are available.

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Abstract

AIM: To investigate the efficacy (survival) and safety of treatments for recurrent hepatocellular carcinoma (HCC) in liver transplantation (LT) patients.

METHODS: Literature search was performed on available online databases without a time limit until January 2015. Clinical studies describing survival after HCC recurrence in LT patients were retrieved for a full-text evaluation. A total of 61 studies were selected: 13 case reports, 41 retrospective case series, and 7 retrospective comparative studies.

RESULTS: Based on all included studies, the mean HCC recurrence rate was 16% of all LTs for HCC. A total of 1021 LT patients experienced HCC recurrence. The median time from LT to HCC recurrence was 13 mo (range 2-132 mo). The majority of patients (67%) presented with HCC extra-hepatic recurrences, involving lung, bone, adrenal gland, peritoneal lymph nodes, and rarely the brain. Overall survival after HCC recurrence was 12.97 mo. Surgical resection of localized HCC recurrence and Sorafenib for controlling systemic spread of HCC recurrence were associated with the higher survival rates (42 and 18 mo, res-

pectively). However, Sorafenib, especially when combined with mTOR, was frequently associated with severe side effects that required dose reduction or discontinuation

CONCLUSION: Management of recurrent HCC in LT patients is challenging and associated with poor prognosis independently of the type of treatment.

Key words: Recurrent hepatocellular carcinoma; Liver transplantation; Tumor recurrence; Surgical resection; Trans-arterial chemoembolization; Sorafenib; Systematic review

Core tip: The present systematic review analyzes the current trends in the management of hepatocellular carcinoma recurrence after liver transplantation (LT). A great variety of treatment options, ranging from surgical resection to systemic therapies (*e.g.*, Sorafenib), are tailored to the different clinical scenarios and aimed to increase patient survival. However, tumor recurrence after LT is still associated with poor prognosis. By summarizing the available literature, the present article provides to clinicians and surgeons the body of knowledge for a better decision-making process and supports researchers in future clinical trials.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer and third leading cause of cancer-related deaths worldwide[1]. In Europe, the age-adjusted incidence rate of HCC is currently 6.2 per 100000 people[2]; however, owing to widespread viral hepatitis infections and nonalcoholic fatty liver disease, HCC incidence and related mortality are expected to increase in western countries over the next 10 years[2-5].

Liver transplantation (LT) is the best treatment option in selected patients for early HCC[6]. When the Milan criteria[7] are fulfilled, the long-term survival following LT for HCC is similar to that following transplantation in patients without HCC[7-9]. However, despite a restrictive patient selection policy, the post-transplant HCC recurrence is reported in up to 25% of cases and drastically affects patient survival[10-13].

Predictors of post-transplant HCC recurrence and prognostic factors have been extensively studied. They have been applied in the selection of candidates for LT and for the construction of models integrating the classic volume-related features (tumor number and maximal size) with histological differentiation, vascular invasion, and, more recently, markers of tumor biological behavior, such as alpha-fetoprotein (AFP) serum level[14-17]. On the contrary, a few well-conducted studies have addressed the management of HCC recurrence in LT patients by describing and comparing clinical outcomes and survival. Currently, the evidence level of recommendations is weak, and there is still a considerable debate about how to treat HCC recurrence in LT patients[6]. Current treatment options include a wide range of therapies, such as surgical resection, transarterial chemoembolization, immunosuppression, and multi-target tyrosine kinases inhibitor (Sorafenib)[18].

The objective of the present systematic review is to summarize and analyze the current available literature in order to evaluate the efficacy and safety of various treatments for HCC recurrence in LT patients. Owing to the lack of international consensus, a systematic approach was chosen to provide an exhaustive report of the clinical experience and current strategies to treat recurrent HCC in LT patients and to support the development of guidelines that will help clinicians in the challenging decision making process.

MATERIALS AND METHODS

The methodological approach included the development of selection criteria, definition of search strategies, assessment of study quality, and abstraction of relevant data. The PRISMA statements checklist for reporting a systematic review was followed[19].

Study inclusion criteria

The study selection criteria were defined before initiating data collection for proper identification of studies eligible for the analysis. All studies in which the primary objective was to evaluate the efficacy, safety, and/or survival of treatments for HCC recurrence in LT patients were retrieved and analyzed if they met the following selection criteria.

Types of study: All types of prospective and retrospective clinical studies, including case reports, were eligible for inclusion without trial duration limitation. Review articles, commentaries, and conference abstracts were not considered.

Types of participants: Adult LT patients with HCC recurrence were eligible. LT from either deceased or living donor were eligible. Patients with hepatocholangiocarcinoma and fibrolamellar hepatocellular

carcinoma were excluded.

Types of interventions: All types of surgical and non-surgical therapies reported in the literature to treat HCC recurrence in LT patients were eligible.

Types of outcome measures: The primary outcome was survival after treatment of recurrence. The secondary outcomes included safety, tolerability, efficacy, and all other possible clinical parameters evaluated in each study.

Literature search strategy

A literature search was performed on the following online databases: MEDLINE (through PubMed), EMBASE, Scopus, Cochrane Oral Health Group Specialized Register, and ProQuest Dissertations and Thesis Database. To increase the probability of identifying all relevant articles, a specific research equation was formulated for each database, using the following keywords and/or MeSH terms: hepatocellular carcinoma, recurrence, recurrent hepatocellular carcinoma, liver transplantation, liver transplant, treatment, therapy, management, and Sorafenib (*i.e.*, Nexavar®). In addition, reference lists from eligible studies and relevant review articles (not included in the systematic review) were crosschecked to identify additional studies. A grey literature search was also performed by using the OpenGrey database. No time limitation was applied. Studies written in English, French, Spanish or Italian and meeting the selection criteria were reviewed.

Study selection and quality assessment

The titles and abstracts of the retrieved studies were independently and blindly screened for relevance by two reviewers (NdeA and MCC). To enhance sensitivity, records were removed only if both reviewers excluded the record at the title screening level. All disagreements were resolved by discussion with a third reviewer (FL). Subsequently, both reviewers performed a full-text analysis of the selected articles. The two reviewers independently assessed the risk of bias using appropriate tools according to the study design. Briefly, the Cochrane criteria described in the Cochrane Handbook for Systematic Reviews of Interventions[20] were applied for RCT, and the Newcastle-Ottawa Scale (NOS)[21] was used for the non-randomized studies. Additionally, the Grading of Recommendations Assessment Development and Evaluation (GRADE) system was used to grade the "body of evidence" merging from this review[22].

Data extraction

Data from the studies included in the systematic review were processed for qualitative and possibly quantitative analyses. Outcome measures (mean values, standard deviation, and ranges) were extracted for each treatment approach. Average survival was calculated as the weighted mean (and standard deviation) of median survivals reported in the included studies. Data from case reports were not pooled into the measurement of the outcomes of interest due to the low level of evidence of this study design.

RESULTS

Literature search and selection

All database searches were performed without time limit until January 2015. Overall, the combined search identified 124 articles (after removing duplicates); of these, 61 were rejected based upon title and abstract evaluation. Out of the remaining 63 articles (which underwent a full-text evaluation), 24 were excluded because they were not pertinent to the review questions, had non-relevant study design, or had language limitations. By reference crosscheck analysis and manual search, we identified 22 additional publications that were included in the total count. Finally, 61 articles were found eligible for the systematic review and were evaluated for both qualitative and quantitative analyses. The flow chart of the study identification and inclusion/exclusion process is shown in Figure 1.

Study characteristics

The selected studies included 13 case reports[23-35], 41 retrospective case series[36-76], and 7 retrospective case-control/comparative studies[77-83]. Studies were conducted in 12 different countries including those in Europe (n = 28), North America (n = 13), Asia, and the Pacific (n = 20). Six (9.8%) studies out of 61 were multicentric. The study time frame ranged from 1987 to 2013. Since the first report published in 1995, a progressive increase in the number of publications was noted (Figure 2).

Based on the included studies, the mean rate of HCC recurrence was an average 16% of all patients receiving LT for HCC. The median time from LT to HCC recurrence was 13 mo (range 2-132 mo). The mean age at HCC recurrence diagnosis was 53.8 years, and 84.3% of affected patients being males. Nearly 51% of LT recipients were classified as beyond the Milan Criteria (upon examination of the explanted liver), and 44.5% of LTs were performed from living donors (LDLT).

Overall, 1021 LT patients presenting with HCC recurrence were treated by different modalities. The majority (67%) were extra-hepatic HCC recurrences involving lung, bone, adrenal gland, peritoneal lymph nodes, and, more rarely, the brain. Only 33% of the described cases were limited to hepatic HCC recurrence at diagnosis. All included studies are summarized in Supplementary file.

Treatment modalities

HCC recurrence was managed with surgical resections, radiofrequency ablation (RFA), microwave ablation,

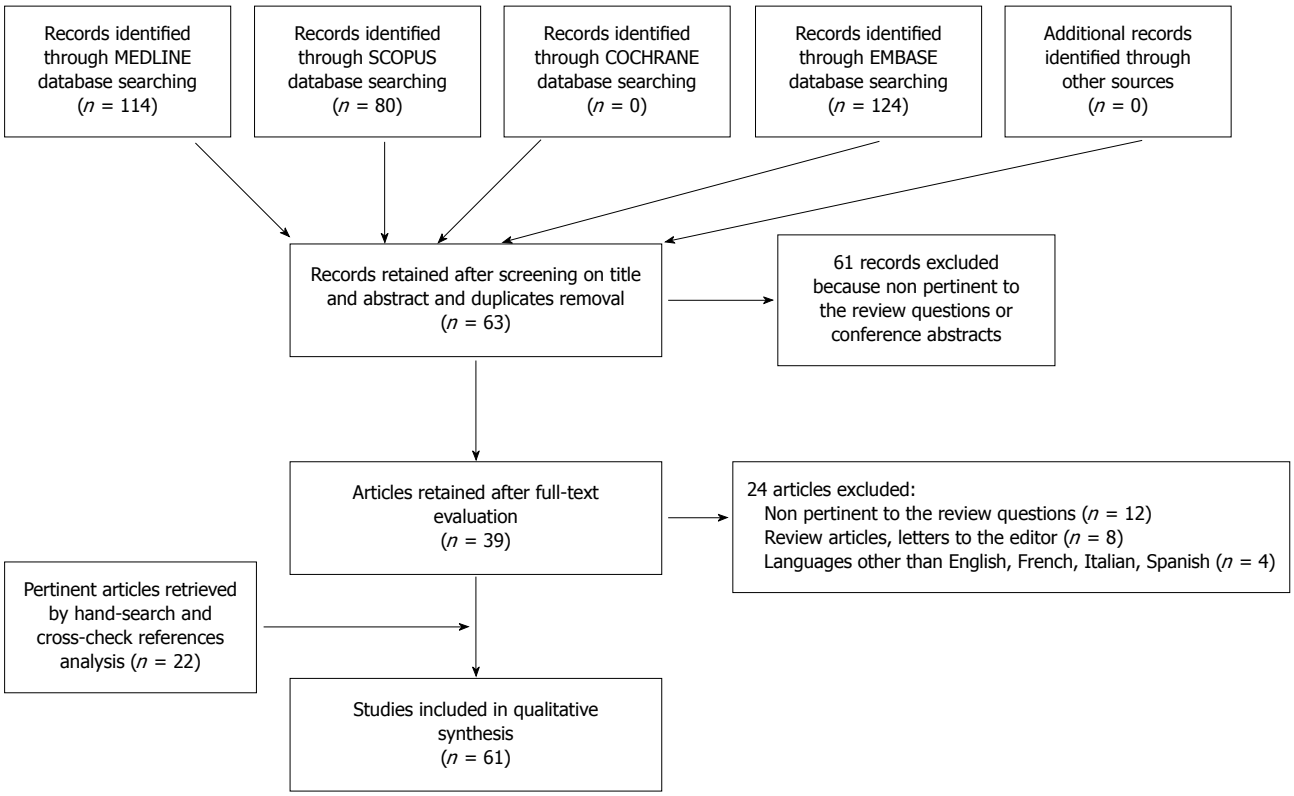

Figure 1 Flow chart of the electronic literature search strategy using MEDLINE, Scopus, EMBASE and other sources. Examples of PubMed Equations: recurrent hepatocellular carcinoma[Title/Abstract] OR recurrent hepatocellular carcinoma[MeSH Terms] OR hepatocellular carcinoma recurrence[Title/Abstract] AND liver transplantation[MeSH Terms] OR liver transplantation[Title/Abstract] OR liver transplant[Title/Abstract] OR liver transplant[MeSH Terms] AND treatment[Title/Abstract] OR therapy[Title/Abstract] OR management[Title/Abstract] OR recurrent hepatocellular carcinoma[Title/Abstract] OR recurrent hepatocellular carcinoma[MeSH Terms] OR hepatocellular carcinoma recurrence[Title/Abstract] AND liver transplantation[MeSH Terms] OR liver transplantation[Title/Abstract] OR liver transplant[Title/Abstract] OR liver transplant[MeSH Terms] AND sorafenib[Title/Abstract] OR sorafenib[MeSH Terms] OR Nexavar[Title/Abstract]) OR Nexavar[MeSH Terms]).

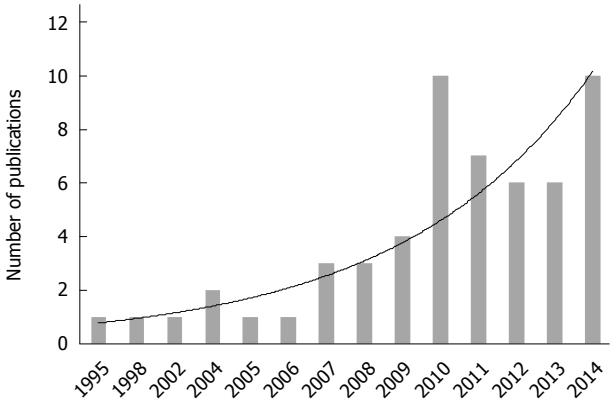

Figure 2 Increasing number of publication on the management of recurrent hepatocellular carcinoma in liver transplantation patients.

percutaneous ethanol injection (PEI), selective internal radiotherapy treatment (SIRT), stereotactic body radiation therapy, brachytherapy, transarterial chemoembolization (TACE), re-transplantation, immunosuppression (*e.g.*, mTOR), systemic chemotherapy, administration of a multi-kinase inhibitor agent (Sorafenib), and best supportive care (BSC). Most of the studies applied multimodality therapies, which usually involved surgery and systemic treatments.

Survival after treatment for HCC recurrence

The overall median survival after HCC recurrence was 12.97 mo (mean range: 0.1-112.5 mo) (Supplementary file). In order to estimate the survival per treatment, the included studies were grouped based on the type of management applied and were considered into the estimation only if they reported the median survival for the specific treatment modality[34,38,39,45,47,50,54,58,63-65,73,74,76-80,84] (Table 1). Among the loco-regional treatments, surgical resection of HCC recurrence was associated with the highest survival (42 mo), whereas in the case of systemic spread of HCC recurrence, a longer survival was related to the use of Sorafenib combined with immunosuppression (mTOR) (18.3 mo). In all related studies, BSC was associated with the lowest survival (3.3 mo) (Table 1).

Efficacy and safety of surgical resection

Surgical resection was performed for either localized intra-hepatic or extra-hepatic HCC recurrence. Resection of isolated HCC recurrence appeared to be safe and effective, with no reported post-operative mortality. The post-operative period was also reported as uneventful in the majority of the study[24,40,71,75,83];

Table 1 Mean survival time after specific treatment modalities for local and systemic hepatocellular carcinoma recurrence in liver transplantation patients

Type of treatment for HCC recurrence in LT patients	No. of patients	Median survival[1] (mo) (weighted mean ± SD)	Ref.
Loco-regional treatments for resectable local recurrence of HCC			
Surgery	27	42 ± 24.45	Bates *et al*[38] Kornberg *et al*[50] Pfiffer *et al*[54] Kim *et al*[47] Chen *et al*[77] Sommacale *et al*[74]
TACE	40	11.2 ± 8.81	Tan *et al*[80] Kim *et al*[47] Pfiffer *et al*[54] Carr[39] Chen *et al*[77] Yamagami *et al*[64]
Systemic treatments for unresectable, advanced, multifocal recurrence of HCC			
Sorafenib	76	12.1 ± 9.95	Tan *et al*[80] Yoon *et al*[65] Pfiffer *et al*[54] Staufer *et al*[76] Sposito *et al*[79] Pfeiffenberger *et al*[78] Alsina *et al*[73]
Sorafenib + mTOR	68	18.2 ± 6.53	Waidmann *et al*[34] Gomez-Martin *et al*[45] Weimann *et al*[63] Staufer *et al*[76] Sotiropoulos *et al*[58]
Systemic chemotherapy	35	5.79 ± 2.7	Lee *et al*[84] Kim *et al*[47]
Best supportive care	54	3.3 ± 2.12	Kim *et al*[47] Pfiffer *et al*[54] Yoon *et al*[82] Sposito *et al*[79]

[1]Weighted mean survival (standard deviation) is calculated from the studies that reported the median survival in months from HCC recurrence diagnosis. TACE: Transarterial chemoembolization; HCC: Hepatocellular carcinoma; LT: Liver transplantation; mTOR: Mammalian target of rapamycin.

one study, however, reported a very high morbidity rate after liver resection for post-LT HCC recurrence[74] (Table 2).

Efficacy and safety of loco-regional therapies

Among the loco-regional therapies for HCC recurrence, TACE was the most frequently reported. Overall, TACE was well tolerated and not associated with major adverse events. Anecdotal reports described SIRT, RFA, and CT guided brachytherapy as effective, well-tolerated and safe loco-regional treatment approaches (Table 3).

Efficacy and safety of Sorafenib: Since 2009, several studies investigated the safety and effectiveness of Sorafenib alone or in combination with modified immunosuppressors, namely Everolimus or Sirolimus (mTOR). As summarized in Table 4, the most common dose of Sorafenib was 400 mg/bid. However, this dosage was rarely tolerated, and dose reduction or discontinuation was reported in the majority of the studies. Overall, 197 patients were treated with Sorafenib: 42.1% of these required dose reduction, whereas 9.6% discontinued the therapy due to intolerable side effects. The most common side effects observed in almost all studies included gastrointestinal symptoms, hand foot skin reactions, hypertension, and fatigue. Six out of 23 studies[26,34,45,53,66,76] reported severe adverse events following the administration of Sorafenib combined with mTOR; among these, 4 cases reported death (Table 4).

Based on the RECIST classification (*Response Evaluation Criteria in Solid Tumors*) or its modified version (mRECIST)[85] used in some studies, Sorafenib appeared to have only a partial effect; indeed, stable disease was observed in the majority of the cases (46.5%), whereas a considerable amount of patients (37%) showed progression of the disease. Only in a minority of cases (5.5%), a complete response to Sorafenib was found.

Study quality assessment: Two reviewers (NdeA and MCC) scored the methodological qualities of the included studies according to the criteria described above. The majority of the studies were case reports or case series without any comparison between different treatments. No RCT was found, therefore the Cochrane criteria were not applied. Only 12 retrospective studies

Table 2 Morbidity and mortality from surgical treatments for recurrent hepatocellular carcinoma in liver transplantation patients

Ref.	No. of patients	Surgical treatments (No. of patients)	Post-operative morbidity	Post-operative mortality
Regalia *et al*[75]	7	Liver resection (2), pulmonary lobectomy (2), omentectomy (1), bone resection (1), skin resection (1)	Uneventful	0
Castroagudìn *et al*[24]	1	Bilateral adrenalectomies in a successive manner	Uneventful	0
Catalano *et al*[40]	2	Liver resection (2)	Uneventful	0
Roayaie *et al*[55]	15	Liver resection (5), pulmonary resection (7), adrenalectomy (2), chest wall resection (1)	NR	0
Bates *et al*[38]	5	Pulmonary lobectomy (4), pulmonary lobectomy + rib resection after pre-transplant tumor biopsy (1)	NR	0
Kwon *et al*[71]	7	Pulmonary resection (7)	Uneventful	0
Marangoni *et al*[52]	4	Liver resection (4)	NS	0
Han *et al*[69]	12	Pulmonary resection (12)	NS	0
Kornberg *et al*[50]	7	Liver resection (2), pulmonary resection (2), cerebral tumor extirpation (1), adrenalectomy (1), chest wall resection after pre-transplant tumor biopsy (1)	NR	0
Valdivieso *et al*[61]	8	Liver resection (2), adrenalectomy (2), abdominal lymph node resection (2), pulmonary resection (2)	NR	0
Kitano *et al*[70]	3	Pulmonary resection (3)		0
Pfiffer *et al*[54]	7	Liver resection (1), extra-hepatic resection (6)	NR	NR
Kim *et al*[47]	3	Left adrenalectomy (1), splenectomy (1), lymph node resection	NR	0
Chen *et al*[77]	2	Pulmonary resection (1), adrenalectomy (1)	NR	0
Hwang *et al*[83]	23	Pulmonary resection (23)	Uneventful	0
Sommacale *et al*[74]	3	Liver resection (3)	100% (renal failure, respiratory sepsis, sub phrenic abscess)	0

NR: Not reported; HCC: Hepatocellular carcinoma; LT: Liver transplantation.

(19.6%) performed statistical comparisons between groups of treatments for HCC recurrence in LT patients. The quality and risk of bias in these studies were assessed according to the Newcastle-Ottawa Criteria and are summarized in Supplementary file. Overall, 3 studies were classified at a low risk of bias, and 9 studies were at high risk of bias.

In addition, the GRADE system was used to enable consistent judgment of the quality of the available evidence included in this systematic review. The quality of the evidence was rated according to the following aspects: study design, study quality, consistency, and directness of results. Fourteen studies[42,47,49,55-57,59,68,72,75,79-81,83] (22.9%) were judged as being of low quality, and the remaining 47 studies[23-41,43-46,50,52-54,58,60-67,69-71,73,74,76-78,82,84,86] had a very low quality of evidence. Of note, the majority of studies were retrospective, which, by definition, are susceptible of major selection bias as well as misclassification or information bias due to the unknown accuracy of record keeping.

DISCUSSION

Despite the stringent selection of LT candidates and measures to control the recipient[16,56,87] and, more recently, donor-related[88] risk factors, post-transplant HCC recurrence is a reality that occurs in average 16% of patients and drastically affects their survival. Treatments for HCC recurrence have been receiving increasing attention in the literature, as shown by the rising number of publications in the recent years. However, there are no randomized clinical trial or large sample prospective studies available on the topic. The present systematic review might provide a better understanding of the clinical experience and thus support the development of treatment strategies tailored for different clinical settings.

As supported by several studies[50,57,59,60,72], the time between LT and HCC recurrence is a key predictor of survival, with worse prognosis associated with early HCC recurrence (within 24 mo). From a physiopathological perspective, early HCC recurrence could occur due to non-detectable extra-hepatic metastases that were present before LT, as well as a consequence of circulating HCC cell clones engrafting and growing in a target organ in the post-transplant period[89]. Conversely, late HCC recurrence appeared to be the consequence of late engrafting of HCC cells that remained latent and less numerous for a longer time post LT[60,89]. In LT patients, immunosuppression may also play a role in the recurrence of HCC[90,91]; however, it remains controversial whether the administration of mTOR as a first line of management after LT can control both the inherent risk of graft rejection and the potential risk of tumor recurrence[6,92,93] or impact on the delay and extension of HCC recurrence. Based on all included studies, the median HCC recurrence time was 13 mo; thus, the majority of HCC recurrence appeared early after LT. It was not possible from the available data to make distinctions and comparisons between early and late HCC recurrence. Although described in some studies, the survival was usually reported for all patients together. Notwithstanding,

Table 3 Efficacy, safety and tolerability of loco-regional treatments for recurrent hepatocellular

Ref.	No. of patients	Treatment	No. of treatments per patient	Efficacy (No.of patients)	Side effects (No. of patients)	Tolerability	Safety
Rivera *et al*[31]	1	SIRT (Y-90)	1	Efficacy demonstrated by tumor necrosis on imaging and decreased AFP level	Intermittent nausea Mild right upper quadrant abdominal pain	Well tolerated	No adverse consequence
Ho *et al*[27]	1	RFA	1	No evidence of local progression and normalization of AFP levels	None	Well tolerated	No adverse consequence
Ko *et al*[49]	28	TACE	2.5	Complete response (3), partial response (11), minimal response (5), stable disease (3), progressive disease (6)	In 17.9% of patients: Nausea, vomiting, diarrhea Hypertension, tachycardia Mild right upper quadrant abdominal pain	Well tolerated	No adverse consequence
Tan *et al*[80]	10	TACE	NR	According to RECIST criteria, partial response (1), stable disease (3), and progressive disease (6)	NR	Well tolerated	No adverse consequence
Carr[39]	6	TACE	8.2	Complete response (1), partial response (2), stable disease (1), progression (2)	Bilirubin toxicity (Grade 2) (1) Granulocyte toxicity (Grade 3) (3)	Well tolerated	No adverse consequence
Chen *et al*[77]	4	TACE	2.8	According to mRECIST criteria, complete or partial response in all patients	None	Well tolerated	No adverse consequence
Cheng *et al*[41]	11	TACE	NR	NR	NR	Well tolerated	No adverse consequence
Zhang *et al*[67]	10	CT 125I guided brachytherapy	3.9	Complete local control of HCC recurrence 72% of patients at 2 yr	Minor displacement of radioactive seeds (2) Mild increase of white blood cell counts (3) Fever (4)	Well tolerated	Hemothorax (1) Hemosputum (3)

CT: Computed tomography; TACE: Transarterial chemoembolization; RFA: Radiofrequency ablation; SIRT: Selective internal radiotherapy treatment; HCC: Hepatocellular carcinoma; LT: Liver transplantation.

early HCC recurrence can be considered a negative prognostic factor for survival[42,47,50,60].

The majority of patients presented as extra-hepatic HCC recurrence at diagnosis, most commonly located at the lung, bone, adrenal gland, and abdominal lymph nodes. Hepatic recurrence, especially late recurrence, might be indicative of *de novo* HCC development from recurrent hepatitis and cirrhosis in the liver graft. However, in the absence of molecular profiling analyses to distinguish recipient origin from donor origin, it is impossible to determinate the nature of HCC recurrence[94]. As it is rarely described, the actual incidence of *de novo* HCC post-LT remains unclear.

Another prognostic factor that emerged from the analysis of the literature is the pattern of HCC recurrence, either as localized, isolated nodule(s) or multifocal metastases. This is shown to impact on patient survival as well as on the choice of treatment strategies. For both isolated hepatic and extra-hepatic metastases, surgical resection appeared to be the best treatment option offering longer survival chance[50,52,59,61,72,75,83]. However, a major selection bias has to be highlighted: LT patients with HCC recurrence undergoing surgery are usually the ones with the best performance status, late recurrence, and most favorable localization allowing resection. Indeed, reported post-operative mortality and morbidity were very low in both resection of grafted liver and other organ metastases (*e.g.*, lung, adrenal gland). Based on the current knowledge, surgery for HCC recurrence is a valuable option if performed in selected patients with curative intents, and, as recommended in a recent consensus conference[6], surgery should be attempted whenever feasible. On the contrary, little is known about re-transplantation for intra-hepatic recurrent HCC, and it is currently considered not appropriate[6].

Selected patients with unresectable but limited HCC recurrence may undergo loco-regional therapy including TACE, SIRT, and RFA, with potential improvement in survival[6]. These treatments appeared to be safe and well tolerated and may be repeated multiple times or combined in a multimodality approach[31,41,49,54,56,68,75,80].

When recurrent HCC is presenting as or becomes systemically spread, systemic treatments are warranted[6]. Among these, systemic chemotherapy

Table 4 Efficacy, safety and tolerability of Sorafenib for recurrent hepatocellular carcinoma in liver transplantation patients

Ref.	*n*	Treatments	Type of immunosuppression	Efficacy (No. of patients)	Most frequent side effects[1]	Tolerability	Safety (No. of patients)
Yeganeh *et al*[35]	1	Sorafenib (400 m bid)	Tacrolimus + mycophenolate mofetil	Complete resolution of his lung lesion	Diarrhea Hand-foot skin reactions	Dose reduction needed (200 mg bid)	No major adverse consequence
Bhoori *et al*[23]	1	Sorafenib (400 mg bid)	Everolimus	50% response according to RECIST criteria modifications after introduction of Everolimus	Hand-foot skin reactions (Grade 1)	Well tolerated	No major adverse consequence
Herden *et al*[26]	1	Sorafenib (200 mg bid)	Cyclosporine A	NR	Nausea Fever up to 39 ℃ Jaundice	Discontinuation after 5 d of treatment	Centrolobular hepatocellular necrosis and lymphoplasmacellular and granulocytic infiltration of portal tracts with significant eosinophilia (hyper allergic drug reaction)
Kim *et al*[48]	9	Sorafenib (200 to 400 mg bid)	Tacrolimus ± Sirolimus	Complete radiographic response (1), stable disease (4), progression (3)	Hand-foot skin reactions Fatigue and anorexia Diarrhea Mucositis	Discontinuation in 5 patients	No major adverse consequence nor deterioration of liver graft function
Tan *et al*[80]	10	Sorafenib (400 mg bid)	Tacrolimus	Stable disease (7), progressive disease (3)	Rash Hypertension Agrypnia Hand-foot skin reaction Diarrhea	Discontinuation in 1 patient	No major adverse consequence
Valdivieso *et al*[61]	5	Sorafenib (400 mg bid)	Everolimus	NR	NR	NR	NR
Wang *et al*[37]	1	Sorafenib (400 mg bid)	Tacrolimus/ Sirolimus	Partial response (after introduction of Sirolimus)	Hand-foot skin reactions	Dose reduction needed (200 mg bid)	No major adverse consequence
Yoon *et al*[65]	13	Sorafenib (400 mg bid)	Calcineurin inhibitors ± mycophenolate mofetil	Stable disease (6)	Chest wall pain Hand-foot skin reactions Neutropenia, thrombocytopenia, anemia Rash Diarrhea	Dose reduction (200 mg bid) in 4 patients	No major adverse consequence
Kim *et al*[28]	1	Sorafenib (200 to 400 mg bid)	Sirolimus	Complete radiologic response	Hand-foot skin reactions Fatigue Mucositis	Dose reduction needed (200 mg bid)	No major adverse consequence
Takahara *et al*[33]	2	Sorafenib (200 to 400 mg bid)	Cyclosporine/ Tacrolimus	Complete response (1)	Hypertension Diarrhea, anorexia	Dose reduction (200 mg bid) for 1 patient	No major adverse consequence
Waidmann *et al*[34]	3	Sorafenib (400 mg bid)	Everolimus/ Sirolimus	Partial response after introduction of mTOR (1), progression (1)	Hand-foot skin reactions (Grade 3) Fatigue (Grade 3)	Discontinuation for 1 patient, dose reduction (200 mg bid) for 1 patient	Death for sepsis and multi-organ failure (1) after 3 wk of Sorafenib treatment
Gomez-Martin *et al*[45]	31	Sorafenib (400 mg bid)	Everolimus	Complete response (1), partial response (1), and stable disease (13)	Mild graft dysfunction Hand-foot skin reactions Asthenia Hypertension Diarrhea Thrombocytopenia	Dose reduction (200-300 mg bid) in 8 patients	Central nervous system hemorrhaging (1), severe biventricular heart failure (1), and upper digestive hemorrhaging (2) leading to death (1)

Sotiropoulos *et al*[58]	14	Sorafenib (400 mg bid)	mTOR	Progression (4)	NR	Discontinuation for 4 patients, dose reduction (100 to 200 mg bid) for 2 patients	NR
Staufer *et al*[76]	13	Sorafenib (400 mg bid)	mTOR/ Cyclosporine A	Partial response (1), stable disease (4), progression (7)	Anemia, leukopenia Hand-foot skin reactions Increase of liver function tests Fatigue	Poor tolerability. Dose reduction (200 mg bid) in 10 patients	Centrolobular hepatocellular necrosis and lymphoplasmacellular infiltration of portal tracts (2) with eosinophilia (2)
Vitale *et al*[62]	10	Sorafenib (400 mg bid)	mTOR/Tacrolimus	According to mRECIST criteria, partial response (2), stable disease (6), progression (2)	Diarrhea Diarrhea Hand-foot skin reactions Fatigue	Dose reduction in 7 patients	No major adverse consequence
Weinmann *et al*[63]	11	Sorafenib (400 mg bid)	Sirolimus	Stable disease (4), progression (7)	Diarrhea Fatigue Nausea Hand-foot skin reactions Hair loss Weight loss	Discontinuation and dose reduction (200 mg bid) for 7 patients	No major adverse consequence
Pfeiffenberger *et al*[78]	8	Sorafenib (400 mg bid)	Tacrolimus/ Tacrolimus + mycophenolate mofetil/Cyclosporin A	Progression (1)	Hand-foot skin reaction Diarrhea	Dose reduction for 6 patients	No major adverse consequence
Sposito *et al*[79]	15	Sorafenib (400 mg bid)	mTOR/ Cyclosporine A/ Tacrolimus	According to RECIST, stable disease (11), partial response (4)	Hand-foot skin reactions Diarrhea Fatigue	Dose reduction (200 mg bid) for 8 patients	No major adverse consequence
Waghray *et al*[81]	17	Sorafenib (400 mg bid)	Sirolimus	Complete response (2), partial response (1), stable disease (2), progression (5)	Diarrhea, nausea Fatigue Increase of liver function tests Hand-foot skin reactions	Dose reduction (100 to 200 mg bid) for 14 patients	No major adverse consequence
Zavaglia *et al*[66]	11	Sorafenib (400 mg bid)	Everolimus/ Cyclosporine A	Partial response (2), stable disease (1), progression (6)	Fatigue, anorexia Hypophosphatemia Diarrhea, nausea, vomiting Hand-foot skin reactions	Discontinuation in 4 patients, dose reduction (200 mg bid) for 7 patients	Massive gastrointestinal bleeding leading to death after 4 mo of Sorafenib + mTOR
Alsina *et al*[73]	9	Sorafenib (400 mg bid)	Tacrolimus/ Cyclosporine A ± mycophenolate mofetil	NR	Hand-foot skin reaction Rash Seizure Skin flushing	Dose reduction for 2 patients	No major adverse consequence
De Simone *et al*[43]	7	Sorafenib (400 mg bid)	Everolimus	According to mRECIST, progression (5)	Hand-foot skin reactions Hypertension Diarrhea Anorexia, asthenia Hoarseness Alopecia	Dose reduction for 3 patients, temporary discontinuation for 2 patients	No major adverse consequence
Perricone *et al*[53]	4	Sorafenib (200 mg bid)	Everolimus	Stable disease (1), progression (3)	Diarrhea (Grade 3)	Discontinuation for 1 patient	Severe diarrhea with progressive worsening of clinical condition, leading to coma then death (1) 4 mo after Sorafenib + mTOR

[1]When available, Sorafenib-related adverse events were graded according to the National Cancer Institute Common Toxicity Criteria (Grade Ⅰ to Ⅴ). RECIST: Response Evaluation Criteria in Solid Tumors; mTOR: Mammalian target of rapamycin; HCC: Hepatocellular carcinoma; LT: Liver transplantation.

(*e.g.*, doxorubicin, or fluoropyrimidine and platinum) demonstrated very limited efficacy in both preventing and controlling HCC recurrence[84], confirming HCC as a low chemo-sensitivity tumor. Little is known about the efficacy of systemic chemotherapy combined with other immunosuppressive agents like mTOR.

In contrast, systemic therapy with Sorafenib has gained significant interest in the recent years and has been the object of several publications assessing its efficacy and safety for the treatment of HCC recurrence in LT patients. Some studies demonstrated that the administration of Sorafenib, with or without mTOR, is associated with improvement of patient survival[45,58,62,63,73,79,81]. However, several studies also reported adverse effects related to Sorafenib, which required dose reduction or discontinuation in a high percentage of patients[26,34,43,45,53,66,76]. Of note, Sorafenib has been associated with major adverse effects (Grade 3 and 4) including five cases of centrolobular hepatocellular necrosis with lymphoplasmacellular and granulocytic infiltration of the portal tracts (with or without eosinophilia)[26,45,76], 1 case of massive gastrointestinal bleeding[66], 1 case of sepsis and multi-organ failure[34], and 1 case of severe diarrhea[53]. The complication of these conditions led to death in 4 patients. Therefore, the risk/benefit and the cost/effectiveness ratios of Sorafenib remain unknown and should be further investigated[95]. However, pooling together the available data, it seems that most of the times, Sorafenib is able to stabilize the disease, even though complete responses are rare. Notwithstanding, a benefit in patient survival post-HCC recurrence has been reported in the majority of the study series[23,34,61,65,73,76,79,80], which was assessed at 12.1 mo for Sorafenib alone and at 18.2 mo for Sorafenib + mTOR. This is highly superior to the 3.3 mo of survival for patients receiving best supportive care[79]. A synergistic effect of Sorafenib + mTOR inhibitors has been advocated by some authors[23,45,79]; however, the current evidence is insufficient to draw definitive conclusions. Moreover, it was not possible to specifically assess the effects of Sorafenib combined with different ongoing immunosuppressive therapies (*e.g.*, calcineurin inhibitors, Tacrolimus).

The use of mTOR has also been proposed as an immunosuppression paradigm to be applied at the time of HCC recurrence[93,96]. This is because mTOR seems to have a strong immunosuppressive activity with concomitant antineoplastic properties[97] that may prevent or control HCC recurrence as well as protect from *de novo* cancers[98].

Managing post-transplant recurrent HCC is a challenging field, as reflected by the highly heterogeneous conditions and treatment strategies encountered in the literature. Since the first report in 1995[30], novel therapies have been introduced; however, international guidelines are still lacking. The available evidence is of rather low quality to sort management paradigms with predictable outcomes. Waiting for a significant progress, the best treatment may likely remain the prevention of HCC recurrence by applying stringent selection criteria for LT candidates and relying on up-to-date imaging and biological assessments, which need to be repeated shortly before transplantation.

However, once HCC recurrence is diagnosed in LT patients, clinicians and surgeons face a difficult decision making process. Recently, Toso *et al*[98] proposed a management algorithm that applies different treatment strategies depending on the localization of the recurrence (hepatic *vs* extra-hepatic) and the feasibility of surgical resection. The present systematic review supports this paradigm, which can be summarized as follows: (1) consider to switch to mTOR or decrease overall immunosuppression; (2) apply surgery whenever feasible; (3) reserve more aggressive approaches for cases with better potential outcomes (*e.g.*, late recurrence); and (4) recur to systemic treatments, namely Sorafenib, for unresectable multifocal disease and HCC re-recurrence.

The present systematic review has several limitations mainly related to the retrospective nature of the included studies, which displayed high heterogeneity in the clinical parameters and outcomes evaluated, missing data, and small sample size. Moreover, external validity of the present results cannot be assured due to the highly selected study populations and specialized centers in which the clinical trials have been performed.

The clinical management of HCC recurrence in LT patients is challenging and is associated with a poor prognosis independently of the type of treatment. Most of the time, a multimodal approach is required to slow down the disease progression. Although the administration of Sorafenib with or without mTOR appears promising, further clinical trials are needed to assess its efficacy and safety. Finally, an international consensus meeting should be advocated in order to assess guidelines and draw up future research directions in the field of HCC recurrence management in LT patients.

COMMENTS

Background

Hepatocellular carcinoma (HCC) recurrence after liver transplantation drastically affects patient survival. Management of HCC recurrence involves a wide range of local and systemic treatment modalities that lack established guidelines.

Research frontiers

The present systematic review provides a better understanding of the clinical experience in the management of post-transplant HCC recurrence. It also highlights the need of developing standardized treatment strategies tailored for different clinical settings.

Innovations and breakthroughs

By means of the systematic approach, this review allows the reader to have an overview of the state of art in the management of post-transplant HCC recurrence, ranging from surgery to systemic therapy. However, this study also highlights that the overall evidence available are low and further well-conducted

studies are needed.

Applications

The clinical management of HCC recurrence in transplanted patients remains challenging and is associated with a poor prognosis independently of the type of treatment. In the near future, research studies need to establish whether the treatment approach has to be chosen based on the underlying etiology of liver disease or HCC recurrence pattern; which are the indications and outcomes of multimodal approaches; which are the efficacy and safety of target therapy combined with immunosuppressant agents. Finally, an international consensus meeting is awaited in order to assess guidelines and clinical recommendations.

Terminology

Sorafenib is an orally active multikinase inhibitor approved for the treatment of advanced HCC. mTOR (*i.e.*, Mammalian target of rapamycin) inhibitors can be used as immunosuppressant agents in transplant patients as well as cancer chemotherapy.

Peer-review

This is an interesting systematic review describing the management of post transplant HCC recurrence.

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CASE REPORT

Case report of primary splenic angiosarcoma with hepatic metastases

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Author contributions: Chen F, Jin HF and Fan YH designed the report; Cai LJ and Zhang ZY collected the patient's clinical data; Lv B contributed to revising the manuscript; Chen F and Jin HF wrote the paper.

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Institutional review board statement: The patient in the case report deceased within 4 weeks of admission to hospital. She was therefore unable to consent to this, but her daughter is available to consent. The case has been discussed with the most senior member of staff in charge of the patient's care who has given consent for this, and consent was obtained for use of accompanying radiological images from the consultant radiologist. The study was reviewed and approved by the First Affiliated Hospital of Zhejiang Chinese Medical University Institutional Review Board.

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

Conflict-of-interest statement: We declare that we have no financial or personal relationships with other individuals or organizations that would inappropriately influence our work. There is no professional or other personal interest of any nature in any product or service.

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Abstract

Primary splenic angiosarcoma (PSA) is the most unusual type of malignancy with early multifocal metastasis through hematogenous spread. PSA is generally believed to originate from splenic sinusoidal vascular endothelium with a high rate of metastasis and to have a poor prognosis. Its etiology and pathogenetic mechanisms have not yet been clearly described. Thus far, only approximately 200 cases have been reported. PSA has variable symptomatology with the potential to present with life-threatening complications. The diagnosis of PSA is challenging; and often late. PSA should be considered in the differential diagnosis of patients with splenomegaly and anemia of unknown etiology. Surgical treatment with splenectomy is considered the only curative intervention for potential long-term disease-free survival. Early diagnosis and treatment are very important. It is important that clinical doctors improve the understanding of PSA. Herein, we report one rare case of PSA with hepatic metastases, along with a review of the current literature.

Key words: Primary splenic angiosarcoma; Hepatic metastases; Rupture; Splenectomy; Case report

Core tip: Primary splenic angiosarcoma (PSA) is an aggressive malignancy with poor prognosis. It has variable symptomatology with the potential to present with life-threatening complications. Its etiology has not yet been established, and its clinical presentation may confuse even experienced physicians. Early diagnosis and treatment are very important. It is important that clinical doctors improve the understanding of PSA. Herein, we report one rare case of PSA with hepatic metastases, along with a review of the current literature.

Chen F, Jin HF, Fan YH, Cai LJ, Zhang ZY, Lv B. Case report of primary splenic angiosarcoma with hepatic metastases. *World J Gastroenterol* 2015; 21(39): 11199-11204 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i39/11199.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i39.11199

INTRODUCTION

PSA is generally believed to originate from splenic sinusoidal vascular endothelium and has a high rate of metastasis and poor prognosis. Thus far, only approximately 200 cases have been reported. Primary splenic angiosarcoma (PSA) has variable symptomatology with the potential to present with life-threatening complications. PSA should be considered in the differential diagnosis of patients with splenomegaly and anemia of unknown etiology. Definitive diagnosis of PSA is challenging and often late. Surgical treatment with splenectomy is considered the only curative intervention for potential long-term disease-free survival. We report herein a rare case of PSA with hepatic metastases and a review of the current literature.

CASE REPORT

A 72-year-old woman presented to our emergency services with right upper quadrant abdominal pain and fatigue for one week. The pain was distending within a sustainable degree lasting for one week. The patient did not present with radiating pain or weight loss. Physical examination was notable for mild jaundice and hepatomegaly (5 cm below the costophrenic margin). A 30-cm-long oblique line of old scars was visible on the left upper quadrant. Blood work at admission revealed a leukocyte count of 4.3 × 10^9/L, with 69.7% neutrophils, 19% lymphocytes, 7.9% monocytes, 1.2% eosinophils and 2.2% basophils, hemoglobin of 70 g/L and a platelet count of 14 × 10^9/L. Evidence of significant coagulopathy, with an D-dimer level of 50.87 mg/L and deranged liver function (ALP 593 U/L, ALT 109 U/L, AST 163 U/L, total bilirubin 96.7 μmol/L, direct bilirubin 67.6 μmol, indirect bilirubin 29.1 U/L, total protein 51.40 g/L, and albumin 27.8 g/L) was noted. Other blood examination results, including ANA, ANCA and tumor markers (CA-199, AFP and CEA), were normal. Imaging studies at admission including abdominal computed tomography (CT) (Figure 1) and magnetic resonance imaging (MRI) (Figure 2) demonstrated obvious hepatomegaly with multiple liver nodules and loss of the spleen. The radiological differential diagnosis included hematological system diseases, such as lymphoma and metastatic carcinoma. A subsequent bone marrow biopsy indicated poor platelet production by megakaryocytes. From her medical history, splenectomy and resection of a left liver tumor after trauma 2 mo previously in another hospital were significant to the diagnosis. Preoperative abdominal CT revealed a massive splenomegaly with hemorrhage, whereas the liver did not exhibit any abnormality. Postoperative pathological findings indicated splenic littoral cell angioma and hepatic cavernous hemangioma. We believed that the multiple nodules of the liver were related to the previous surgery. To confirm the diagnosis, we conducted a multidisciplinary case discussion and rechecked the postoperative pathological section. Histology showed spindled vascular proliferation and area of necrosis (Figure 3). A primary splenic angiosarcoma and hepatic cavernous hemangioma were detected. Immunohistochemical staining of the spleen showed that the lesion was positive for CD31, CD34, F8, and Vim, partially positive for CD68 and CD8, and negative for P53, SMA and CK. The Ki67 index was 20% higher than normal. Postoperative thrombosis, which expended platelets, could explain the thrombocytopenia. Due to the patient's thrombocytopenia, the risk of performing a liver biopsy was extremely high. Therefore a liver biopsy examination was not performed. Based on the immunohistochemical staining, rapid development of disease, clinical and radiological findings, a primary splenic angiosarcoma with hepatic metastases was finally diagnosed. The patient passed away within four weeks after admission.

DISCUSSION

PSA is an aggressive malignancy with a rare incidence of 0.14-0.23 cases per million. The half year survival rate is only 20%[1-3]. The mean age at presentation ranges from 50 to 79 years, with a slight male preponderance but no genetic predisposition[1,4]. This aggressive malignant neoplasm is commonly observed in adults, but can also be observed in pediatric groups[5,6]. The disease was first reported in 1879, with only 200 cases currently reported in the literature, largely as isolated case reports[7,8].

The pathogenesis of this tumor remains unclear.

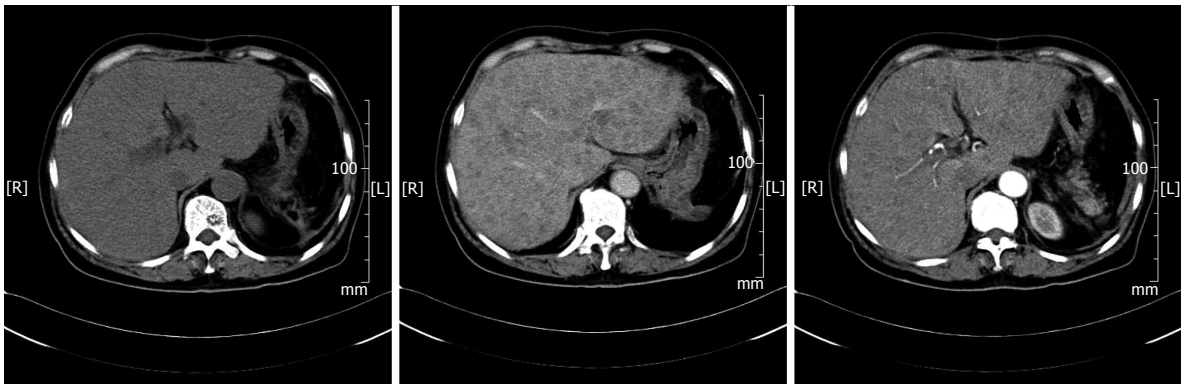

Figure 1 Contrast-enhanced computed tomography images showing hepatomegaly and multiple liver nodules with slight reinforcement and spleen loss.

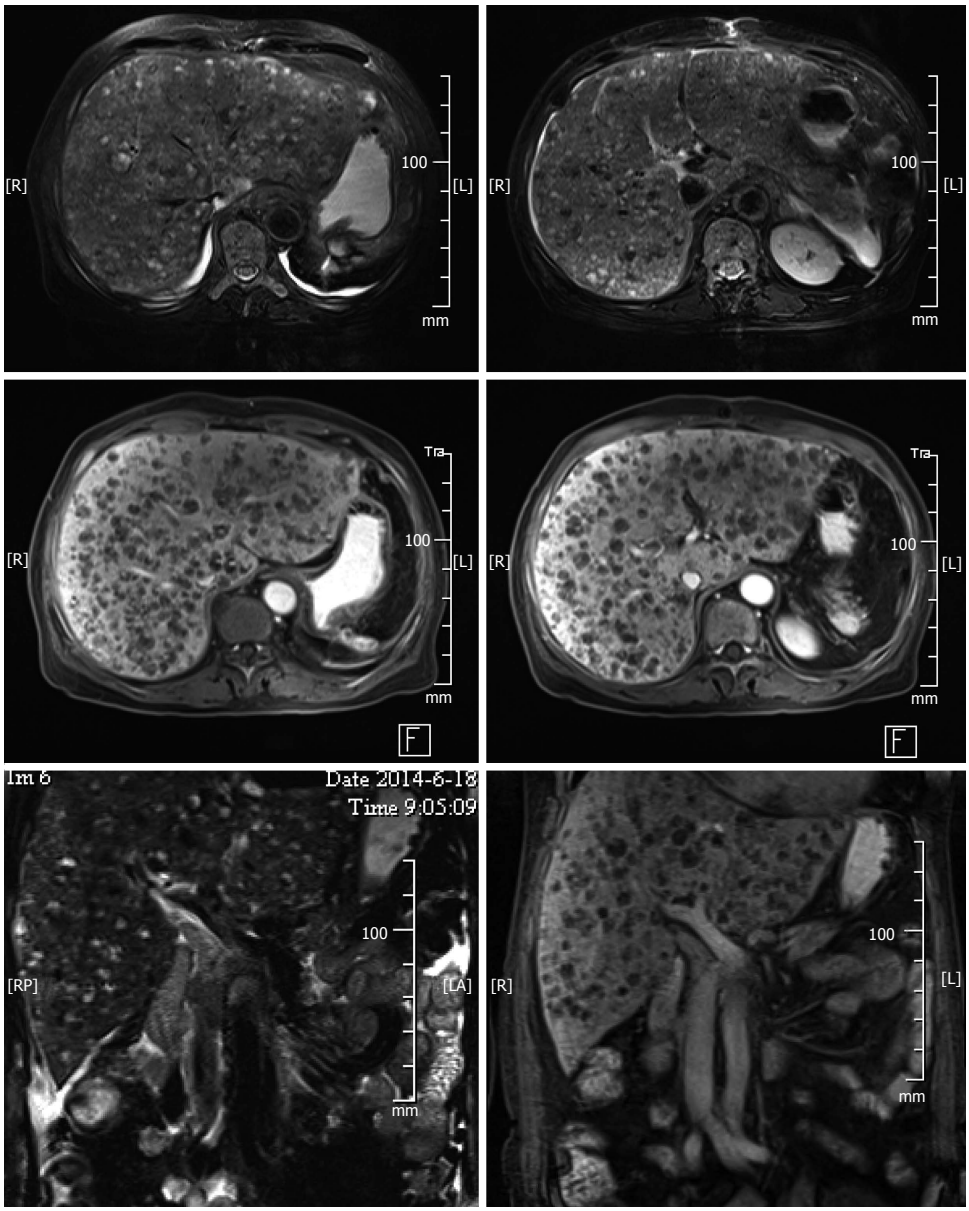

Figure 2 Contrast-enhanced abdominal MR + MRCP showing hepatomegaly and multiple liver nodules without any reinforcement.

For every type of angiosarcoma, thorium dioxide, vinyl chloride, arsenic and chemotherapy for lymphoma or radiation therapy for other malignancies[7,9] have been considered as causative factors. However, no clear relationship between these factors and splenic angiosarcoma has been established. Benign splenic

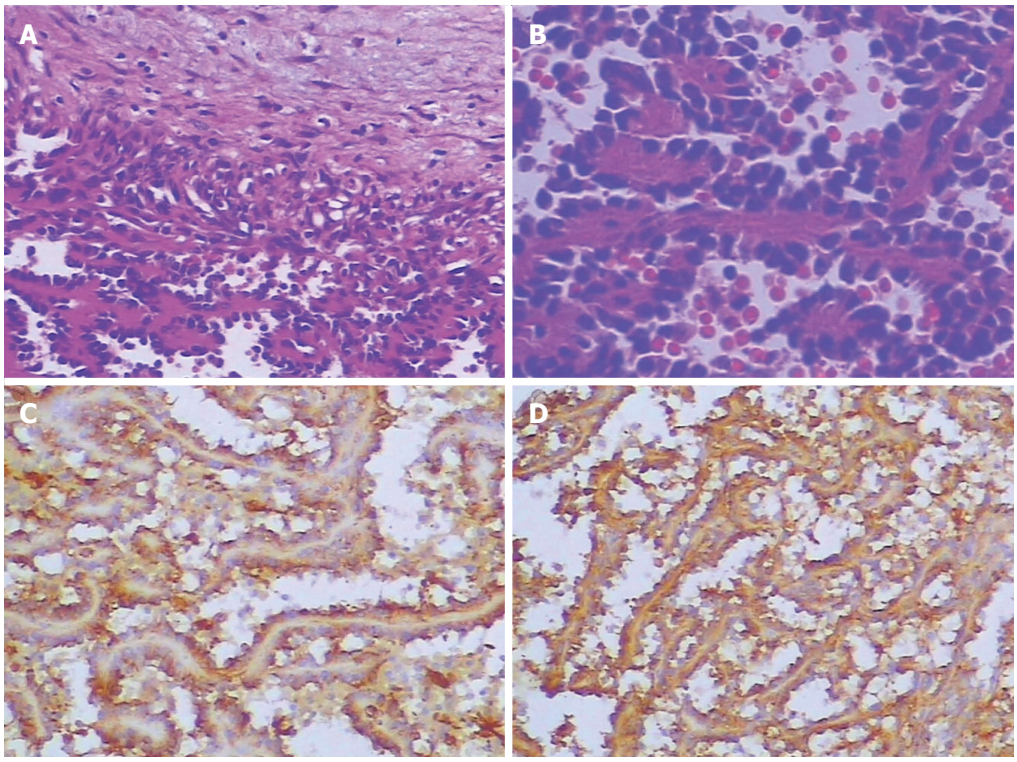

Figure 3 Histological chrematistics of angiosarcoma. A: Histological analysis showing spindled vascular proliferation and area of necrosis (HE staining, × 200); B: Histological analysis showing atypical spindle cells with few mitotic figures (HE staining, × 400); C: Immunohistochemical analysis showing the lesion stained positively for CD31 (× 400); D: Immunohistochemical analysis showing the lesion stained positively for F8 (× 400).

tumors, such as hemangiomas or hemangioendotheliomas, may act as a precursor to splenic angiosarcoma[10,11]. However, there is no evidence that these factors were involved in this patient.

Clinical presentation is nonspecific and may vary from asymptomatic diseases revealed by investigations for unrelated reasons to splenic rupture and lethal hemorrhage[12-14]. Over 75% of patients presented with left upper abdominal pain in one series[15], making it one of the most common presenting symptoms. Other possible complaints include fatigue, anorexia, and weight loss. High temperature, as an associated finding, has been observed in nearly 10% of PSA patients. On physical examination, in addition to splenomegaly as the most consistent sign[7,16], hepatomegaly and a palpable left upper quadrant mass can often be revealed. Blood anomalies, such as anemia and thrombocytopenia, are the most common laboratory abnormalities[17], as in our case, but schistocytes and echinocytes are also common[18]. Spontaneous splenic rupture is observed in 13%-32% of patients presenting with acute abdominal pain[4]. In our case, the patient underwent a splenectomy for traumatic rupture and presented hepatic metastatic cancer as the first manifestation. We considered postoperative thrombosis as the cause of her thrombocytopenia.

Traumatic rupture of angiosarcoma in the spleen is associated with the worst prognosis, with an immediate risk of death from hypovolemic shock and disseminated intravascular coagulopathy. Furthermore, it increases the risk of peritoneal dissemination and hematogenous spread. Reported rates of metastasis range from 69%-100%[4,7,19]. Further, the postoperative metastasis rate remains high. Similar to other forms of angiosarcomas, splenic angiosarcoma commonly has early multifocal metastasis through hematogenous spread[20-22]. Metastasis has been reported by hematogenous routing to the liver, lung, lymph nodes, bone and ovaries[23,24]. In our case, the previous surgery may have accelerated the metastasis, which led to the deterioration.

Imaging modalities are useful for establishing a splenomegaly diagnosis, but are not the determinants of a diagnosis. Specifically, ultrasound, CT and MRI all display supportive evidence of splenomegaly; the most common findings on ultrasound are solitary or multiple, solid and cystic mass lesions with heterogeneous echotexture. On CT imaging, splenic enlargement in the presence of a heterogeneous mass is observed in 60% of cases[25,26]. Contrast CT scanning may reveal non-enhanced areas due to necrosis or enhancement with a blush suggesting active bleeding. There may also be punctuate or widespread calcification. CT imaging is valuable for both diagnosis and acute assessment of complications. Additionally, angiosarcomas may exhibit peripheral or heterogeneous enhancement similar to that of hepatic cavernous hemangiomas. On MRI, both T1-weighted and T2-weighted images show ill-defined nodular lesions with increased or decreased signal intensity, which is related to necrosis or the

presence of hemorrhage or fibrosis within the tumor, respectively[25].

The histologic appearance and immunohistochemical analysis of splenic angiosarcoma may be the gold standard for diagnosing the tumor. This tumor had typical features of angiosarcoma, including vasoformative architecture, highly pleomorphic tumor cells with irregular, hyperchromatic and prominent nucleoli, and some mitotic figures. The tumor exhibited "biphasic" immunoreactivity for vascular and histiocytic markers[5]. Immunohistochemically, pathologists always search for at least two vascular proliferation markers (CD31, CD34, and factor Ⅷ) and at least one histiocytic differentiation marker (lysozyme and/or CD68)[7]. Mark *et al*[27] found that histological appearance or grade was not related to clinical outcome because well-differentiated tumors can behave aggressively. Naka *et al*[28] conducted a multivariate analysis of 55 angiosarcoma cases and found that tumor size, mode of treatment, and mitotic count were independent prognostic factors. Splenectomy is the preferred treatment for localized disease. Montemayor *et al*[23] found that patients had a longer survival time if splenectomy was performed prior to rupture compared with after rupture (14.4 mo *vs* 4.4 mo). There is no specific chemotherapeutic regimen for treating splenic angiosarcoma. Recently, Hara *et al*[29] reported the use of autologous peripheral blood stem cell transplantation combined with high-dose chemotherapy in splenic angiosarcoma. We suggest that older people may attach great importance to the annual medical examination. The longest survival case was a 7-year-old boy reported by Jun-Te Hsu. The boy retained disease-free at 14.8 years after surgery[30].

In conclusion, primary angiosarcoma of the spleen is an aggressive disease that often presents with metastatic disease. Surgery is the only potentially long-term therapeutic option. Early diagnosis and treatment are very important for prognosis. It is important that clinical doctors improve the understanding of PSA.

COMMENTS

Case characteristics

A 72-year-old woman with a history of splenectomy and resection of hepatic cavernous hemangioma after trauma two months prior to presenting at our emergency services with right upper quadrant abdominal pain and fatigue for one week.

Clinical diagnosis

Mild jaundice and obvious hepatomegaly.

Differential diagnosis

Hematological system diseases and metastatic carcinoma.

Laboratory diagnosis

WBC 4.3 × 10^9/L; HGB 70 g/L; PLT 14 × 10^9/L; D-dimer 50.87 mg/L; ALP 593 U/L; ALT 109 U/L; AST 163 U/L; total bilirubin 96.7μmol/l; direct bilirubin 67.6μmol; indirect bilirubin 29.1 U/L; total protein 51.40 g/L; albumin 27.8 g/L; ANA, ANCA and tumor markers (CA-199, AFP and CEA) were within normal limits.

Imaging diagnosis

Abdominal computed tomography and magnetic resonance imaging demonstrated obvious hepatomegaly with multiple liver nodules and spleen loss.

Pathological diagnosis

Immunohistochemical staining of the spleen indicated that the lesion was positive for CD31, CD34, F8, and Vim, partially positive for CD68 and CD8, and negative for P53, SMA and CK. The Ki67 index was 20% higher than normal.

Treatment

The patient was treated with the best supportive treatment.

Related reports

Primary splenic angiosarcoma is an aggressive malignancy with poor prognosis and we must improve the understanding of this rare disease.

Term explanation

Immunohistochemical staining is based on antigen-antibody reactions to detect whether there is a target antigen in cells or tissue.

Experiences and lessons

This case report presents a case of PSA to improve understanding.

Peer-review

This is a case report on primary angiosarcoma of the spleen with hepatic metastases.

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