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Contents

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AIM AND SCOPE

World Journal of Hepatology (*World J Hepatol*, *WJH*, online ISSN 1948-5182, DOI: 10.4254), is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

WJH covers topics concerning liver biology/pathology, cirrhosis and its complications, liver fibrosis, liver failure, portal hypertension, hepatitis B and C and inflammatory disorders, steatohepatitis and metabolic liver disease, hepatocellular carcinoma, biliary tract disease, autoimmune disease, cholestatic and biliary disease, transplantation, genetics, epidemiology, microbiology, molecular and cell biology, nutrition, geriatric and pediatric hepatology, diagnosis and screening, endoscopy, imaging, and advanced technology. Priority publication will be given to articles concerning diagnosis and treatment of hepatology diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

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TOPIC HIGHLIGHT

WJH 6th Anniversary Special Issues (1): Management of hepatocellular carcinoma

Role of anti-angiogenesis therapy in the management of hepatocellular carcinoma: The jury is still out

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Abstract

As the leading cause of disease-related deaths, cancer is a major public health threat worldwide. Surgical resection is still the first-line therapy for patients with early-stage cancers. However, postoperative relapse and metastasis remain the cause of 90% of deaths of patients with solid organ malignancies, including hepatocellular carcinoma (HCC). With the rapid development of molecular biology techniques in recent years, molecularly targeted therapies using monoclonal antibodies, small molecules, and vaccines have become a milestone in cancer therapeutic by significantly improving the survival of cancer patients, and have opened a window of hope for patients with advanced cancer. Hypervascularization is a major characteristic of HCC. It has been reported that anti-angiogenic treatments, which inhibit blood vessel formation, are highly effective for treating HCC. However, the efficacy and safety of anti-angiogenesis therapies remain controversial. Sorafenib is an oral multikinase inhibitor with anti-proliferative and anti-angiogenic effects and is the first molecular target drug approved for the treatment of advanced HCC. While sorafenib has shown promising therapeutic effects, substantial evidence of primary and acquired resistance to sorafenib has been reported. Numerous clinical trials have been conducted to evaluate a large number of molecularly targeted drugs for treating HCC, but most drugs exhibited less efficacy and/or higher toxicity compared to sorafenib. Therefore, understanding the mechanism(s) underlying sorafenib resistance of cancer cells is highlighted for efficiently treating HCC. This concise review aims to provide an overview of anti-angiogenesis therapy in the management of HCC and to discuss the common mechanisms of resistance to anti-angiogenesis therapies.

Key words: Hepatocellular carcinoma; Management; Molecularly targeted therapy; Anti-angiogenesis; Sorafenib

Core tip: Hepatocellular carcinoma (HCC) is a devastating disease with a high mortality rate. For a long period of time, no effective treatment options are available for patients with advanced HCC. During the last decade, molecularly targeted therapies have been introduced into the treatment of advanced HCC. However, the efficacy and safety of molecularly targeted therapies remain controversial. In addition, primary or acquired drug resistance limits the activity of molecularly targeted agents, but the underlying mechanisms have not been fully understood. This concise review aims to

provide an overview of anti-angiogenesis therapy in the treatment of HCC.

Sun H, Zhu MS, Wu WR, Shi XD, Xu LB. Role of anti-angiogenesis therapy in the management of hepatocellular carcinoma: The jury is still out. *World J Hepatol* 2014; 6(12): 830-835 Available from: URL: http://www.wjgnet.com/1948-5182/full/v6/i12/830.htm DOI: http://dx.doi.org/10.4254/wjh.v6.i12.830

INTRODUCTION

Primary liver cancer (PLC) is one of the most common malignancies and the second leading cause of cancer-related deaths around the world. Hepatocellular carcinoma (HCC), the most common type of PLC, accounts for approximate 90% of PLC cases in most countries. In addition, HCC is the 5th and 7th most common cancer in males and females, respectively. The worldwide incidence of HCC is increasing partially due to the rising number of infections caused by hepatitis B virus or hepatitis C virus[1-3]. Recently, while the diagnosis of HCC has been remarkably improved with the use of noninvasive imaging tests, a large number of patients were still diagnosed at the advanced stage due to the lack of symptoms during early stages and the rapid progression of cancer cells[4,5].

The management of HCC depends mainly on tumor stage and liver function reserve. Currently, curative treatments such as surgical resection, liver transplantation, and local ablation can significantly improve the survival of HCC patients at the early stage[2,6]. However for a long period of time, no effective treatment options are available for patients with advanced HCC or who progressed into an advanced stage after other treatments failed. In recent years, molecularly targeted therapies using monoclonal antibodies, small molecules, and vaccines have been widely studied in cancer managements. Given that HCC is a highly vascularized tumor, anti-angiogenic treatments might be highly efficient for the treatment of HCC by inhibiting the formation of blood vessels in cancer tissues through small molecules[7-9].

RESISTANCE TO ANTI-ANGIOGENIC DRUGS OF HCC CELLS

As an oral multikinase inhibitor, sorafenib has both anti-proliferative and anti-angiogenic effects on tumors through blocking Raf and vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) receptor tyrosine kinase signaling. Sorafenib is the first molecular target drug approved for the treatment of advanced HCC. A phase 3, randomized, double-blind, placebo-controlled, multicenter study was performed in 2008 in 21 Western countries to evaluate the effects of sorafenib on the treatment of HCC. This study showed that sorafenib prolonged the median survival and the time to radiologic progression by approximately 3 mo in advanced HCC patients[10,11]. Cheng *et al*[12] also reported that sorafenib was effective for advanced HCC and was well tolerated in HCC patients from the Asia-Pacific region. In addition, high safety and well-tolerance of sorafenib have been reported in a large phase 4 study including over 1500 patients with unresectable HCC[13,14]. Therefore, sorafenib has been established as the standard first-line monotherapy for patients with advanced HCC[9,15-17]. However, the efficacies of current anti-angiogenesis therapies are still far from satisfactory (Table 1). Currently, the median survival time of HCC patients who received sorafenib treatment is not longer than 1 year even after many years of research[18].

Resistance to molecularly targeted agents including sorafenib is a major reason causing the failure of anticancer therapies (Table 2)[17,19,20]. Primary resistance is observed in some HCC patients who are initially not susceptible to sorafenib therapy due to intrinsic indifference. After long-term exposure, tumor cells may gradually become resistant and/or less susceptible to sorafenib, leading to acquired resistance[17]. Both primary and acquired resistance to sorafenib has been commonly reported in HCC patients[21]. Ezzoukhry *et al*[22] found that HCC cells exhibited different susceptibilities to sorafenib. For example, some HCC cell lines such as Hep3B and SNU-449 were inherently resistant to sorafenib. The authors also showed that activation of the epidermal growth factor receptor (EGFR) was a possible determinant of inherent resistance of HCC cells to sorafenib. In an *in vitro* study, Zhang *et al*[23] showed that phosphorylated extracellular signal-regulated kinase was a potential predictor of sorafenib sensitivity in HCC. Similarly, Blivet-Van Eggelpoël *et al*[21] demonstrated that EGFR and human epidermal growth factor receptor-3 reduced the susceptibility of HCC cells to sorafenib.

The exact molecular mechanisms underlying the acquired resistance to sorafenib are largely unknown[17]. In 2011, Chen *et al*[24] reported that activation of the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway mediates acquired resistance to sorafenib in HCC cells. Xia *et al*[25] also showed that activation of the transforming growth factor beta-and PI3K/Akt-signaling pathways led to acquired resistance to sorafenib in HCC cells. Recently, a number of studies provided evidence showing that many mechanisms such as cancer stem cells[26-29], epithelial-mesenchymal transition[25,26,28-30], autophagy[31-34], and microenvironment (hypoxic, inflammation, and cytokines)[35-38] were involved in the acquired resistance to anti-angiogenesis therapies of HCC[17,39]. In addition, Zhai *et al*[17] suggested in a review article that sorafenib could simultaneously or sequentially activate the addiction switches and compensatory pathways when its targets were silenced, leading to acquired resistance. Taken together, the exact mechanisms of sorafenib resistance have not been fully elucidated. Therefore, further studies should be conducted to clarify the biological mechanisms, which may further improve the therapeutic effects of sorafenib.

Table 1 Clinical studies on anti-angiogenesis therapy of hepatocellular carcinoma included in this review

Ref.	Year	Phase	Investigational drug	Outcome
Llovet *et al*[10]	2008	Phase 3	Sorafenib	Increased survival
Cheng *et al*[12]	2009	Phase 3	Sorafenib	Increased survival
Lencioni *et al*[13]	2012	Phase 4	Sorafenib	High safety
Lencioni *et al*[14]	2014	Phase 4	Sorafenib	High safety
Johnson *et al*[40]	2013	Phase 3	Brivanib	Less well-tolerated
Cheng *et al*[41]	2013	Phase 3	Sunitinib	Significantly inferior than sorafenib
Zhu *et al*[42]	2012	Phase 3	Sorafenib plus erlotinib	No survival benefit
Llovet *et al*[43]	2013	Phase 3	Brivanib after sorafenib failed	No survival benefit
Zhu *et al*[44]	2014	Phase 3	Everolimus after sorafenib failed	No survival benefit

Table 2 Studies on the mechanisms of anti-angiogenesis therapy resistance in hepatocellular carcinoma

Ref.	Year	Investigational drug	Pathways/genes involved	Effects
Blivet-Van Eggelpoël *et al*[21]	2012	Sorafenib	EGFR and HER-3	Restrict cell response
Ezzoukhry *et al*[22]	2012	Sorafenib	EGFR	Potential determinant of primary resistance
Zhang *et al*[23]	2009	Sorafenib	pERK	Potential biomarker for sensitivity prediction
Chen *et al*[24]	2011	Sorafenib	PI3K/Akt	Mediates acquired resistance
Xia *et al*[25]	2013	Sorafenib	TGF-β-and PI3K/Akt	Mediates acquired resistance
Chen *et al*[26]	2011	Sorafenib	EMT and hedgehog signaling	Drug resistance
Xin *et al*[27]	2013	Sorafenib	CSCs	Drug resistance
Chow *et al*[28]	2013	Sorafenib	EMT	Acquired resistance
Fernando *et al*[29]	2014	Sorafenib	TGF-β pathway	Prediction of low susceptibility
Huang *et al*[30]	2013	Sorafenib	EMT	Drug resistance
Shi *et al*[31]	2011	Sorafenib	Autophagy	Drug resistance
Shimizu *et al*[32]	2012	Sorafenib	Autophagy	Impair antitumor effects
Zhai *et al*[33]	2014	Sorafenib	Autophagy	Acquired resistance
Liu *et al*[34]	2013	Sorafenib	Autophagy	Facilitates resistance
Liang *et al*[36]	2013	Sorafenib	Hypoxia	Drug resistance
Mao *et al*[38]	2014	Sorafenib	microRNA-193b	Enhances cell response
Ebos *et al*[46]	2009	Sunitinib	VEGFR/PDGFR	Accelerate metastasis and decrease overall survival
Pàez-Ribes *et al*[47]	2009	Sunitinib	VEGFR/PDGFR	Increase local invasion and distant metastasis
Xiong *et al*[50]	2009	Sorafenib	TECs	Drug resistance
Li *et al*[53]	2011	Bevacizumab	Dll4-notch signaling	Drug resistance

EGFR: Epidermal growth factor receptor; HER-3: Human epidermal growth factor receptor-3; pERK: Phosphorylated extracellular signal-regulated kinase; PI3K: Phosphatidylinositol 3-kinase; TGF-β: Transforming growth factor beta; EMT: Epithelial-mesenchymal transition; CSCs: Cancer stem cells; TECs: Tumor-derived endothelial cells; VEGFR: Vascular endothelial growth factor receptors; PDGFR: Platelet-derived growth factor receptors; Dll4: Delta-like ligand 4.

The discovery and development of sorafenib have paved the way to the development of new anti-angiogenesis drugs for advanced HCC or for whom sorafenib failed. More recently, many clinical trials are conducted all over the world, but the problem still exists. Due to good results from preclinical and early-phase studies, some other molecularly targeted drugs have been applied as the second-line treatment for advanced HCC when sorafenib treatment fails. In a number of large-scale randomized phase 3 trials, unfortunately, none of them have shown survival benefits in the first-line (brivanib, sunitinib, erlotinib, and linifanib[40-42]) or second-line (brivanib[43], everolimus[44]) setting after sorafenib progression[18,45].

Furthermore, it was proposed that anti-angiogenic therapies may cause tumor progression and metastasis. Ebos *et al*[46] reported that sunitinib (a VEGF receptors/PDGF receptors kinase inhibitor) promoted tumor growth and metastasis after a short-term application. Similarly, Pàez-Ribes *et al*[47] demonstrated that application of angiogenic inhibitors targeting the VEGF signaling pathway elicit malignant progression of tumors to increased local invasion, lymphatic and distant metastasis. Recently, Chow *et al*[28] reported that advanced HCC patients with acquired resistance to sorafenib might have enhanced tumor growth properties or metastatic potentials. Therefore, understanding the molecular mechanisms underlying anti-angiogenesis therapy resistance may allow us to identify key molecular targets for efficient anti-angiogenesis therapy.

NEW MECHANISMS OF RESISTANCE TO ANTI-ANGIOGENIC DRUGS

During the last five years, increasing evidence suggested that tumor-derived endothelial cells (TECs), which exhibit distinct histologic appearance compared to normal endothelial cells (NECs), may contribute to the resistance of anti-angiogenic therapies[48,49]. In 2009, Xiong *et al*[50] reported that TECs in human HCC tissues had higher angiogenic capacity and sorafenib resistance than NECs.

Some researchers have concluded that TECs can acquire molecular cytogenetic abnormalities in tumor microenvironment; however, the molecular mechanisms underlying the resistance of TECs to anti-angiogenic therapies remain largely unknown. Attempts to resolve this dilemma have resulted in the discovery of transdifferentiation of tumor cells to vascular endothelial cells. In 2010, Wang *et al*[51] and Ricci-Vitiani *et al*[52] provided strong evidence showing that a number of TECs that contribute to blood vessels in glioblastoma were transdifferentiated from tumor stem-like cells. Wang *et al*[51] also showed that blocking the VEGF/VEGFR2 signaling pathway inhibited the maturation of tumor endothelial progenitors into endothelia but not the differentiation of tumor stem-like cells into endothelial progenitors, while the initial differentiation of tumor stem-like cells to endothelial progenitor cells was regulated by Notch1. Consistently, Li *et al*[53] reported that Delta-like ligand 4 (Dll4; a novel Notch ligand)-Notch signaling mediated the resistance to VEGF inhibitor bevacizumab and Dll4-expressing tumors were resistant to a VEGFR targeting multikinase inhibitor *in vivo*. Furthermore, it has also been shown that Dll4-mediated Notch signaling played a central role in active vascularization[54] and blockade of Dll4 resulted in tumor growth inhibition even for tumors resistant to anti-VEGF treatments[55].

CONCLUSION

In summary, sorafenib is still the only approved drug for the therapy of advanced HCC. However, the long-term survival benefit from sorafenib treatment is relatively limited. Some other anti-angiogenesis drugs have been evaluated preclinically and clinically for the treatment of HCC, but their effects were not satisfactory. Therefore, identification of novel anti-angiogenic drugs and improvement of the currently available anti-angiogenesis therapies are highlighted for the treatment of HCC.

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TOPIC HIGHLIGHT

WJH 6th Anniversary Special Issues (2): Hepatocellular carcinoma

Role of hepatectomy for recurrent or initially unresectable hepatocellular carcinoma

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Abstract

As a result of donor shortage and high postoperative morbidity and mortality after liver transplantation, hepatectomy is the most widely applicable and reliable option for curative treatment of hepatocellular carcinoma (HCC). Because intrahepatic tumor recurrence is frequent after loco-regional therapy, repeated treatments are advocated provided background liver function is maintained. Among treatments including local ablation and transarterial chemoembolization, hepatectomy provides the best long-term outcomes, but studies comparing hepatectomy with other nonsurgical treatments require careful review for selection bias. In patients with initially unresectable HCC, transarterial chemo-or radio-embolization, and/or systemic chemotherapy can down-stage the tumor and conversion to resectable HCC is achieved in approximately 20% of patients. However, complete response is rare, and salvage hepatectomy is essential to help prolong patients' survival. To counter the short recurrence-free survival, excellent overall survival is obtained by combining and repeating different treatments. It is important to recognize hepatectomy as a complement, rather than a contraindication, to other nonsurgical treatments in a multidisciplinary approach for patients with HCC, including recurrent or unresectable tumors.

Key words: Hepatocellular carcinoma; Hepatectomy; Repeat hepatectomy; Conversion therapy; Multidisciplinary treatment

Core tip: Previous studies comparing hepatectomy with other nonsurgical treatments for hepatocellular carcinoma (HCC) evaluated which provided superior survival benefit. However, considering the high recurrence rate after curative loco-regional treatment, and limited indications for hepatectomy because of background liver damage, it is important to recognize hepatectomy as a complement to other nonsurgical treatment, rather than a contraindication. A multidisciplinary approach combining and repeating different treatments prolongs patients' survival with HCC, including those with recurrent or initially unresectable tumors.

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INTRODUCTION

Liver transplantation is the most promising strategy for radical treatment for hepatocellular carcinoma (HCC) because it eradicates both the tumors and the background damaged liver; hepatectomy is second. However, high perioperative morbidity and mortality, and a shortage of donors limit application of liver transplantation. Poon *et al*[1,2] reported that although the risk of postoperative

Table 1 Repeat resection rate, 5-year recurrence-free survival rate, and overall survival rate after repeat hepatectomy in previous studies

Ref.	Year	Number of primary hepatectomy	Number of second hepatectomy/HCC recurrence after primary hepatectomy	5-yr recurrence free survival after repeat hepatectomy	5-yr overall survival after repeat hepatectomy
Poon *et al*[4]	1999	244	11/105 (10%)	NA	69%
Nakajima *et al*[7]	2001	94	12/57 (21%)	Not reached	52%
Sugimachi *et al*[8]	2001	474	78/300 (26%)	NA	47.50%
Minagawa *et al*[9]	2003	334	56/183 (31%)	17%	56%
Chen *et al*[5]	2004	627	34/286 (12%)	NA	56.80%
Taura *et al*[10]	2006	610	55/465 (12%)	NA	NA
Itamoto *et al*[11]	2007	483	70/279 (25%)	10%	50%
Shimada *et al*[12]	2007	319	13/211 (6%)	NA	25%
Tralhão *et al*[6]	2007	190	16/97 (19%)	NA	31%
Liang *et al*[13]	2008	NA	73/853 (9%)	10.50%	27.60%
Choi *et al*[14]	2008	353	9/97 (9%)	NA	78%
Wu *et al*[15]	2009	1177	149/641(23%)	31.80%	56.40%
Kishi *et al*[16]	2011	221	8/134 (6%)	NA	37.50%
Huang *et al*[17]	2012	NA	82/NA	8.20%	22.40%
Tsujita *et al*[18]	2012	NA	112/NA	NA	67.30%
Yamashita *et al*[19]	2013	791	163/308 (53%)	29%	60%

HCC: Hepatocellular carcinoma; NA: Not assessed.

tumor recurrence was low after transplantation, the long-term prognosis after transplantation was comparable to patients who underwent hepatectomy among patients with Child-Pugh class A background liver disease. Therefore, hepatectomy remains a reliable and widely applicable surgical treatment; however, the main limitation is that it is not indicated in patients with impaired liver function resulting from cirrhosis irrespective of the etiology of the liver disease. Multimodal therapy combining nonsurgical treatments including local ablation and transarterial chemoembolization (TACE) with hepatectomy and/or liver transplantation have been advocated for recurrent HCC, multinodular HCC, or initially unresectable HCC. This review was aimed to evaluate the role of hepatectomy among the various treatments for recurrent or advanced HCC.

Hepatectomy for recurrent HCC following local treatment

Because HCC usually develops in the injured liver, tumors frequently recur even after curative local treatment. The incidence of intrahepatic recurrence within 2 years after primary hepatic resection is 70%[3]. However, because recurrences occur most commonly in the remnant liver, comprising 85%-90% of initial recurrence sites[3], repeat hepatectomy or other local treatment is indicated. In general, treatments are selected based on the same criteria as the primary HCC. Several studies compared the results of repeat hepatectomy with nonsurgical treatment and showed that repeat hepatectomy was associated with a better prognosis[4-6]. However, these studies were retrospective analyses and may have included the selection bias that the repeat hepatectomy group usually included patients with better background liver function and less multinodular tumors. Repeat hepatic resection is indicated for only a limited proportion of patients (6%-53%) and the 5-year overall survival after second hepatectomy is reported as 22%-78%[4-19]. The repeat resection rate, 5-year recurrence-free survival rate, and overall survival rate after second hepatectomy in these studies are summarized in Table 1. The difference in the survival rate would probably have been influenced by the difference in the background liver damage, types of recurrence, and tumoral factors such as size, number, and vascular invasions, but precise assessment was difficult due to the insufficient data. A small number of studies reported the outcomes after a third or fourth hepatectomy[15,19]. In the two series evaluating the outcomes of 1117 and 791 patients who underwent primary hepatectomy for HCC, a second, third, and fourth hepatectomy was performed in 23% (149/641) and 53% (163/308), 37% (35/96), and 65% (36/55), and 27% (8/30) and 69% (9/13) of the patients with recurrence, respectively. Five-year overall survival after a second and third hepatectomy was 56% and 59% in Wu *et al*'s[15] series and 60% and 43% in Yamashita *et al*'s[19] series, respectively. Factors related to both primary and recurrent tumors such as tumor size, number, and vascular invasion and also the degree of background liver damage as assessed by Child-Pugh class, indocyanine green retention rate, or platelet counts were reported as prognostic predictors. Recurrence-free interval and/or type of recurrence, multicentric occurrence or intrahepatic metastases[17,20], were also commonly reported to be prognostic predictors in several studies. Intrahepatic metastases usually occur *via* the portal vein, and are therefore associated with portal vein invasion. Distinction of them is important because intrahepatic recurrence is associated with malignant behavior compared to multicentric occurrence. Differentiation is possible by histopathological examination as defined by the Liver Cancer Study Group of Japan (Table 2)[21] but there is no established method to differentiate intrahepatic metastases *vs* multicentric occurrence preoperatively, an issue requiring further research.

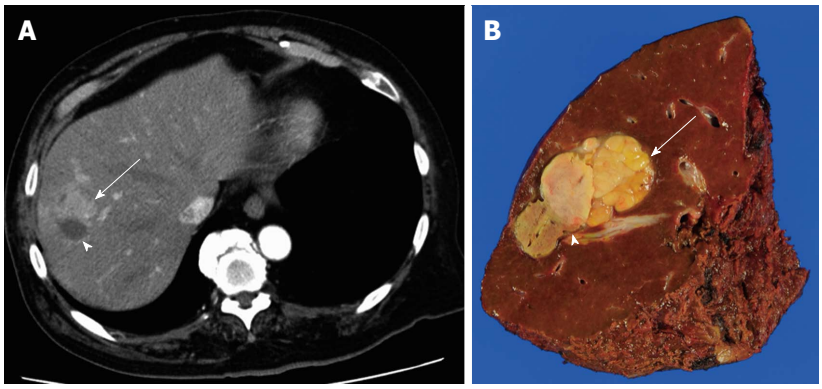

Figure 1 Recurrent hepatocellular carcinoma adjacent to a radiofrequency ablation scar. A: Computed tomography showing the tumor with unclear borders with arterial enhancement (arrow) adjacent to the scar (arrowhead); B: Cut surface of the resected specimen showing the recurrent tumor (arrow) and radiofrequency ablation scar (arrowhead).

Table 2 Three types of definition of intrahepatic metastases by the Liver Cancer Study Group of Japan[21]

	Definition
1	Tumors clearly growing from portal vein tumor thrombi
2	Tumors surrounding a large main tumor with multiple satellite nodules
3	A small solitary tumor that is near the main tumor and histologically similar to or less differentiated than the main tumor

It is important that hepatectomy and other local treatments be considered complementary and not exclusive. The dissociation between low recurrence-free survival and rather high overall survival shown in Table 1 reflects the slow progression of the disease and the importance of repeating treatment, usually TACE. Repeating locoregional treatment such as ethanol injection (PEI), radiofrequency ablation (RFA), or TACE, for intrahepatic recurrence prolongs patient survival[10,22-25], and provides a comparable prognosis after RFA compared with repeat hepatectomy[7,12,13,24]. Taura *et al*[10] compared the long-term outcomes of 610 patients with HCC who underwent hepatectomy before 1990 and after 1991. There was no change in the disease-free survival (early *vs* late period, 28% *vs* 26%, respectively, at 5 years), but survival after tumor recurrence increased significantly in the later period (12% *vs* 22% at 5 years) and overall survival also improved (39% *vs* 58% at 5 years). The authors concluded that increased application of RFA to solitary intrahepatic recurrence, which was the most common type of recurrence, contributed to the improved prognosis[10]. Kishi *et al*[16] reported that the number rather than the type of treatment for tumor recurrence was associated with prolonged survival.

As was referred in the beginning of the introduction, liver transplantation is the most promising, and salvage liver transplantation for recurrent HCC, which have been reported with 5-year survival rate of 54%-61% could be a choice of treatment because these figures were comparable with that after primary liver transplantation for HCC that was 59%-72%[26-29]. However, shortage of donor organ, expensive medical costs, and contraindication for elderly patients preclude popularization of this strategy. Indication for salvage transplantation have not been established, but various factors including recurrence free survival, microvascular involvement, satellite nodules, as well as tumor number and size at the time of primary hepatectomy and/or transplantation should be considered. Further, intention-to-treat analyses comparing patients who underwent hepatectomy with liver cirrhosis of potentially eligible for transplantation and patients listed for primary liver transplantation showed comparable overall (5-year survival; hepatectomy *vs* listed for transplantation; 66% *vs* 58%; P = NS) and disease-free (41% *vs* 54%: P = NS) survival mainly due to the influence of waiting period[30]. Another intention-to treat analysis also showed the limited value of salvage transplantation with only 28% of transplantability rate and comparable prognosis with the patients with liver resection[31].

Salvage hepatectomy for refractory HCC after other local treatment

Here, the term "refractory HCC" is defined as HCC recognized as remnant, unresponsive, or locally recurred tumor at the site treated with locoregional treatment such as ablation or TACE. The indications for hepatectomy are dictated by the degree of background liver damage, while the indications for RFA are limited less by the degree of liver damage and more by tumor size and location, especially with respect to major vascular structures. We occasionally experience difficult complete resection after local recurrence or remnant HCC after RFA because of unclear tumor borders (Figure 1). Several studies have shown that locally recurrent HCCs after RFA were more invasive because of lower tumor differentiation grade, capsule invasion, and vascular invasion, resulting in the need for extensive liver resection with increased operation time and blood loss[32-36]. In such cases, repeat RFA is rarely indicated and salvage hepatectomy should be the first-choice treatment. The mechanism of aggressive tumor behavior is not clear. Increased intratumoral pressure by RFA may favor intravascular tumor spread[37,38]. Diffi-

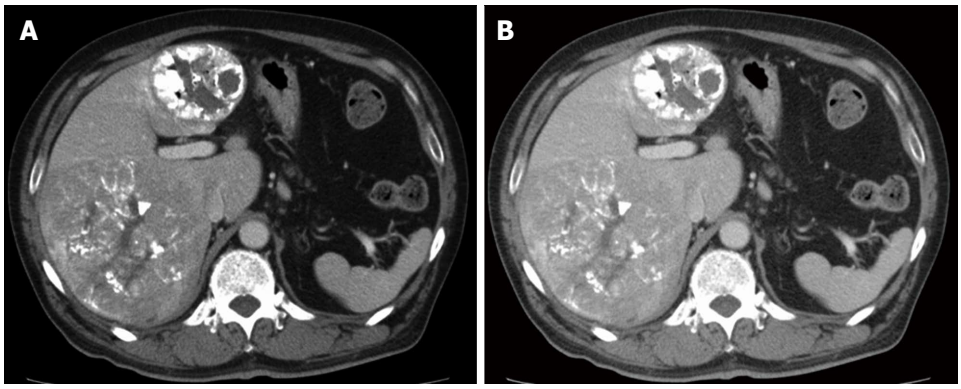

Figure 2 Multinodular hepatocellular carcinomas. Transarterial chemoembolization achieved complete response in one tumor in segment Ⅳ with accumulation of lipiodol showing no arterial enhancement in contrast to the other tumor in segments Ⅵ and Ⅶ that was enhanced in the arterial phase (A) and washed out in the portal phase (B).

culty in early diagnosis of recurrence because of blended necrotic and active areas without a clear delineation may also be a factor[34]. Although recurrence after salvage hepatectomy for these recurrent tumors is frequent, with 5-year recurrence-free survival of 0%-33%, 5-year overall survival is reported as 43%-67%[34-36]. Whether surgical resection or RFA should be selected for HCC that are amenable to both treatments is a controversial issue[39,40], and which is better is still in debate. In a randomized controlled trial by Huang *et al*[41] comparing surgical resection and RFA in patients with HCC meeting the Milan criteria[42], 115 patients were enrolled in each group and both recurrence-free and overall survival was better in the resection group (resection *vs* RFA: 5-year recurrence-free survival, 51.3% *vs* 28.7%, $P = 0.017$; 5-year overall survival, 75.7% *vs* 54.8%, $P = 0.001$)[41]. Hasegawa *et al*[43] reported the results of a Japanese nationwide survey comparing the results of surgical resection, RFA, and PEI in patients with no more than three HCC tumors and with none over 3 cm. A total of 12968 patients with 5361, 5548, and 2059 patients undergoing surgical resection, RFA, and PEI, respectively, were analyzed, and the 5-year recurrence was 63.8%, 71.7%, and 76.9%, respectively (surgical resection *vs* RFA, $P = 0.0001$; RFA *vs* PEI, $P = 0.0001$) and the 5-year overall survival was 71.1%, 61.1%, and 56.3%, respectively (surgical resection *vs* RFA, $P = 0.0001$; RFA *vs* PEI, $P = 0.005$). Although these were the outcomes for the treatment of primary HCC and there have been no established evidence suggesting which of the hepatectomy or ablation is better first choice for recurrent HCC, these results suggest that surgical resection should be selected as a first-line treatment for HCC that is amenable to either surgical resection or ablation, and curative resection should be attempted for local recurrence after ablation for as long as possible.

In the treatment of multinodular HCC, surgical resection can be complementary with other nonsurgical therapies to obtain good long-term prognosis even though TACE is usually indicated for multinodular HCC, rather than surgical resection. The guidelines for HCC treatment from the American Association for the Study of Liver Diseases and the European Association for the Study of the Liver[44,45], based on the Barcelona Clinic Liver Cancer criteria[46] recommend hepatic resection only for patients with solitary tumor without portal hypertension. In the Japanese guidelines, surgical resection is indicated for patients with up to three tumors. For four or more tumors, TACE or transarterial infusion is indicated as the first-choice treatment[47]. We occasionally experience multinodular HCCs treated with repeated TACE showing complete necrosis of a large proportion of the tumors with a small number of remnant viable tumors (Figure 2). It is still unclear whether salvage hepatic resection of the remaining viable tumors is beneficial. A small number of studies have shown benefits with a multimodal approach by combining hepatic resection with simultaneous ablation[48] or reduction surgery followed by ablation and adjuvant TACE or arterial infusion therapy[49]. However, these were retrospective studies with a small number of patients and the details of the exact number of tumors were not provided. Furthermore, differentiation between intrahepatic metastasis and multicentric occurrence is important, as discussed earlier, and criteria as to the number of nodules indicated for hepatectomy remains unclear.

Hepatectomy for down-staged HCC for initially unresectable tumors

In contrast to colorectal liver metastases, in which systemic chemotherapy and/or hepatic artery infusion chemotherapy can convert the unresectable tumor to resectable in > 40% of patients[50-52], HCC conversion therapy has not been established.

Yao *et al*[53] proposed the University of California, San Francisco down-staging protocol inclusion criteria for liver transplantation as: (1) one lesion > 5 cm and up to 8 cm; (2) two to three lesions with at least one lesion > 3 cm and not exceeding 5 cm, with a total tumor diameter up to 8 cm; or (3) four to five lesions with none > 3 cm, with a total tumor diameter up to 8 cm. The authors reported that down-staging was successful in 43/61 patients (71%) and 35 patients underwent liver transplantation with a 4-year survival after transplantation

of 92%[53]. Lei *et al*[54] applied the criteria to hepatectomy and reported the outcomes of 66 of 102 patients (59%) with successful down-staging by TACE and/or RFA. Of the 66 patients, 31 and 35 patients underwent liver transplantation and hepatectomy, respectively, and both recurrence-free (68% and 60% at 5 years, respectively) and overall survival (77% and 69% at 5 years, respectively) were comparable[54]. TACE and/or hepatic artery infusion therapy is usually used as the down-staging treatment. The conversion rate from unresectable to resectable HCC by these modalities was reported as 13%-18%, with a 5-year survival of 49%-56%[55,56].

In contrast to colorectal liver metastases, in which pathologic response is correlated with the prognosis after curative hepatectomy[57], such correlation was not necessarily confirmed in patients with HCC. Of note, Ravaioli *et al*[58] reported that incomplete necrosis by TACE was an independent predictor of poor recurrence-free survival after liver transplantation. Furthermore, several studies showed that preoperative TACE was associated with an increased risk of extrahepatic metastases[59-61]. This might be explained by Adachi *et al*'s[62] hypothesis that viable HCC cells are less firmly attached and likely to spill into the bloodstream during intraoperative manipulation after incomplete response to TACE. Because complete necrosis is rarely obtained, especially for large tumors, the routine application of preoperative TACE for resectable HCC is not recommended. However, based on results showing that a proportion of patients can undergo curative resection following down-staging by TACE and obtain long-term survival, aggressive loco-regional treatment to attempt curative resection should be adopted in patients with initially unresectable HCCs.

The development of other treatment strategies for unresectable HCC such as radioembolization by yttrium-90[63] or systemic treatment combining cisplatin/interferon α-2b/doxorubicin/fluorouracil (PIAF)[64] may increase the rate of conversion. Lau *et al*[65] reported that 49 of 285 patients (17%) underwent salvage surgery following down-staging by intra-arterial yttrium-90 microspheres or PIAF for initially unresectable HCC and obtained a 5-year survival rate of 57%. Notably, 8 of the 49 patients had extrahepatic metastases initially and these patients also obtained long-term survival with a 5-year survival rate > 40% and neither the extension of the disease nor the degree of tumor pathologic response was associated with the prognosis. Although relatively high response rates are obtained with PIAF, frequent adverse events such as neutropenia and thrombocytopenia preclude wide application, especially in patients with cirrhosis[64,66]. In a recent study by Kaseb *et al*[67], an independent predictor of an objective response to PIAF was the use of five or more cycles. The authors suggested that patient selection is important because only responding patients will have an improved prognosis with curative hepatectomy.

To discuss the issue of conversion, it should be noted that the definition of "unresectable" cannot be unanimous and differ according to extension of the tumor, background liver function, and surgeons' judgments. It is also important to recognize that "technically" and "oncologically optimally" resectable are not necessarily the same. It is, however, certain that conversion rate for HCC is still unsatisfactory and the all reports referred above are retrospective studies with small number of patients. Further development of effective treatment for downstaging is expected.

CONCLUSION

Although hepatectomy is indicated for only a small proportion of patients with recurrent or down-staged HCC after primary treatment, an excellent prognosis is obtained if curative resection is achieved, especially for tumors with a multicentric occurrence pattern, rather than intrahepatic metastases. Preoperative differentiation of the two patterns is a future research issue. Even in initially unresectable HCCs, hepatectomy plays a key role in a multidisciplinary approach.

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TOPIC HIGHLIGHT

WJH 6th Anniversary Special Issues (2): Hepatocellular carcinoma

Transarterial chemoembolization for hepatocellular carcinoma: A review of techniques

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Abstract

Hepatocellular carcinoma (HCC) is one of the most common malignant diseases worldwide. While curative therapies, including resection, liver transplantation, and percutaneous ablation (percutaneous ethanol injection and radiofrequency ablation), are applicable for only a portion of the HCC population, transcatheter arterial chemoembolization (TACE) has been recognized as an effective palliative treatment option for patients with advanced HCC. TACE is also used even for single HCCs in which it is difficult to perform surgical resection or locoregional treatment due to systemic co-morbidities or anatomical problems. TACE has become widely adopted in the treatment of HCC. By using computed tomography-angiography, TACE is capable of performing diagnosis and treatment at the same time. Furthermore, TACE plays an important role in the multidisciplinary treatment for HCC when combined with other treatment. In this review, we first discuss the history of TACE, and then review the previous findings about techniques of achieving a locoregional treatment effect (liver infarction treatment, *e.g.*, ultra-selective TACE, balloon-occluded TACE), and the use of TACE as a drug delivery system for anti-cancer agents (palliative, *e.g.*, platinum complex agents, drug-eluting beads) for multiple lesions.

Key words: Hepatocellular carcinoma; Transcatheter arterial chemoembolization; Balloon-occluded transcatheter arterial chemoembolization; Drug-eluting bead

Core tip: Transcatheter arterial chemoembolization (TACE) has become widely adopted in the treatment of hepatocellular carcinoma (HCC). By using computed tomography-angiography, TACE is capable of performing diagnosis and treatment at the same time. Furthermore, TACE plays an important role in the multidisciplinary treatment for HCC when combined with other treatment. In this review, we first discuss the history of TACE, and then review the previous findings about techniques of achieving a locoregional treatment effect (liver infarction treatment, *e.g.*, ultra-selective TACE, balloon-occluded TACE), and the use of TACE as a drug delivery system for anti-cancer agents (palliative, *e.g.*, platinum complex agents, drug-eluting beads) for multiple lesions.

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INTRODUCTION

Hepatocellular carcinoma (HCC) accounts for one-third

of cancer-related deaths worldwide, and has become the fourth leading cause of cancer death in Japan and the seventh leading cause of cancer death in the United States. In recent years, liver cancer deaths have decreased due to remarkable progress in the treatment of viral hepatitis in Japan, while HCC deaths remain high in the United States[1].

Underlying liver disease is present in most HCC cases. Development of HCC in a healthy liver is rare; the majority of patients who develop HCC have a background of chronic hepatitis/cirrhosis viral hepatitis, alcohol abuse, and/or non-alcoholic steatohepatitis. HCC frequently recurs after primary treatment due to the underlying liver disease[2,3].

With advances in diagnostic imaging and treatment in recent years, adaptation of radical treatment strategies such as surgical resection and radiofrequency ablation therapy is increasing. However, even in cases in whom curative treatment is selected as initial treatment, a high recurrence rate due to multi-centric carcinogenesis and intrahepatic metastasis makes it difficult for cure to truly be achieved. Transarterial chemoembolization (TACE) has been widely performed as a treatment for multifocal HCC in patients in whom curative treatment is difficult to perform[4-11].

In the Barcelona Clinic Liver Cancer staging system, TACE is indicated for patients with intermediate-stage HCC (four or more tumors), and in the 2010 Japan Society of Hepatology consensus-based treatment algorithm for HCC, TACE is recommended for patients with a Child-Pugh score A or B, tumor diameter of more than 3 cm, or four or more tumors. However, in real clinical conditions, TACE is selected even for single HCCs in which it is difficult to perform surgical resection or locoregional treatment due to systemic co-morbidities or anatomical problems[12,13].

TACE has been widely adopted in the treatment of HCC. Through the use of computed tomography (CT)-angiography, diagnosis and TACE can be performed at the same time. TACE also plays an important role in the multidisciplinary treatment of HCC as it is often combined with other treatments (*e.g.*, with radiofrequency ablation, with percutaneous ethanol injection, with radiation therapy).

In this review, we discuss the history of TACE and review previous findings about techniques for achieving a locoregional treatment effect (liver infarction treatment) and the use of TACE as a drug delivery system for anti-cancer agents (palliative) for multiple lesions.

CHANGES IN HEPATIC ARTERY CHEMOEMBOLIZATION FOR HCC

TACE induces tumor necrosis through "starvation tactics". It takes advantage of the fact that advanced HCCs are fed only by the hepatic artery and is intended to embolize the distal portion of the hepatic artery. The liver receives blood from the portal vein and hepatic artery at a ratio of 3:1 in the normal liver. Although this ratio varies in cirrhosis, the cirrhotic liver still receives blood flow from both of these vessels. In contrast, classical HCC (moderately-differentiated type) tumors receive nutritional blood flow through the hepatic artery only, and do not depend on portal vein blood flow. By utilizing this property of HCC, TACE was developed by Yamada *et al*[4]. TACE has become widely used for the treatment of HCC since the 1980s. Embolization using adriamycin or mitomycin C and gelatin sponges has been carried out since the first half of the 1980s. Intraarterial injection of lipiodol with anti-cancer drugs before embolic agent results in enhanced embolic effects[14,15]. It also became apparent that using a water-in-oil type emulsion is highly effective for embolization and tumor uptake, and this method is also widely used[16].

Microcatheter insertion into the first three to four branches of the hepatic artery became easily available starting around 1990. Through the use of microcatheter injection of lipiodol into the peripheral branches of the hepatic artery, segmental TACE/subsegmental TACE became a standard treatment. Segmental TACE/subsegmental TACE allows for strong locoregional embolization while stopping the portal blood flow, thereby improving the local treatment effects of TACE[17,18]. In the 2000s, platinum complex agents became available, and treatment effects could be obtained even in HCCs that developed TACE resistance through repeat TACE[19]. Cone beam CT and flat-panel detectors have advanced imaging as they enable more accurate TACE treatment[20,21].

CONVENTIONAL TACE

Intra-tumor concentrations of drugs (particularly polymer drugs) are much higher than those of normal tissue and blood due to the characteristics of blood vessels in solid tumors[14]. In hypervascular HCC, blood returns to the sinusoidal or portal vein; in addition, HCC tissue does not have associated lymph vessels. These features allow stasis of viscous liquid such as lipiodol in the sinusoidal or portal vein in or around HCCs. Nakamura *et al*[22] reported that liver necrosis occurs following injection of lipiodol into the hepatic artery until it is visualized in the portal vein branch. Based on this discovery, Uchida *et al*[15] and Matsui *et al*[18] developed segmental and subsegmental TACE[15,18,22].

The use of water-soluble anti-cancer drugs along with lipiodol as water-in-oil type therapy has been reported to be good for distribution of anti-cancer drugs in HCC[16,23]. Therapy involving selective infusion into tumor vessels of an anti-cancer drug/lipiodol mixture and an embolic agent (gelatin sponge) is generally called conventional TACE (cTACE), and it is widely used as standard treatment worldwide (Table 1).

In 1983 Yamada *et al*[4] reported a 1-year survival rate of 44% for TACE. The 3-year survival rate of segmental TACE reported by Uchida *et al*[15] in 1990 was 67%, Matsui *et al*[18] reported a 4-year survival rate of 67% in

Table 1 Summary of key prospective trials and retrospective studies for the treatment of hepatocellular carcinoma

	Ref.	Year	Analysis	No. of patients	Objective response (%)	Overall survival (%)		
						1 yr	2 yr	3 yr
cTACE								
	Llovet *et al*[7]	2002	Prospective	40	35 (at 6 mo)	82	63	29
	Lo *et al*[8]	2002	Prospective	40	39 (at 3 mo)	57	31	26
	Takayasu *et al*[10]	2006	Prospective	8510	NA	82	63	47
DEB-TACE								
	Lammer *et al*[52]	2010	Prospective	102	51.6 (at 6 mo)	NA	NA	NA
	Sacco *et al*[54]	2011	Prospective	33	100 (at 1 mo)	NA	86.8	NA
	Song *et al*[57]	2012	Retrospective	60	81.6 (at 3 mo)	88	NA	NA
	Wiggermann *et al*[58]	2011	Retrospective	22	22.7 (at 8 mo)	70	NA	NA

TACE: Transcatheter arterial chemoembolization; cTACE: Conventional TACE; DEB: Drug-eluting bead; NA: Not available.

1993, and Takayasu *et al*[24] reported a 3-year survival rate of 77% using interventional radiology (IVR)-CT in subsegmental TACE in 2001. Thus, therapeutic outcomes of TACE have improved rapidly along with advances in TACE techniques, drugs, microcatheters, and the adaption of IVR-CT[25].

ULTRA-SELECTIVE TACE

It has recently become possible to insert microcatheters into the distal hepatic artery more safely due to progress in microcatheter and guidewire technology. Ultra-selective TACE aims to achieve a local therapeutic effect through liver infarction. This technique involves insertion of a microcatheter selectively into a peripheral rather than subsegmental branch (subsubsegment artery), thereby wedging the tumor-feeding vessels, and then injecting lipiodol under high pressure into the tumor and surrounding sinusoids.

The local recurrence rate of ultra-selective TACE has been reported to be 7.9% at 12 mo and 17.7% at 24 mo[26]. Ultra-selective TACE allows injection of lipiodol even into hypovascular lesions in well-differentiated HCC, and is reported to have a local control rate of 53.2% in such cases[27].

With respect to pathological background, in a study of patients who underwent liver resection after undergoing ultra-selective TACE through peripheral branches, necrosis of the tumor as well as the surrounding liver parenchyma was observed[28]. With the spread of fine microcatheters as a treatment aimed at liver infarction, injection of lipiodol in the peripheral rather than the subsegmental branches is becoming a standard treatment[29].

BALLOON-OCCLUDED TACE

Irie *et al*[30] reported in 2008 that better lipiodol deposition was obtained by performing selective TACE while preventing the backflow of embolic material proximally using a micro-balloon catheter, called balloon-occluded TACE (B-TACE)[30]. In conventional TACE, lipiodol suspended with an anti-cancer drug is present in the bloodstream. The blood flow may slow before sufficient lipiodol and drug have reached the tumor, and may even stop flowing. This may occur because when the arterial blood flow is reduced, the backflow of blood to the tumor occurs from the sinusoidal and portal veins. In addition, lipiodol inflow restriction to the normal liver parenchyma is caused by a reduction in peripheral arterial pressure.

In B-TACE, hemodynamic changes caused by balloon occlusion reduce the arterial blood flow by closing the hepatic artery, thereby pushing lipiodol into the tumor under high pressure and enabling the drug to be intensively administered to the tumor, allowing for an enhanced therapeutic effect. In recent years, since micro-balloon catheters with small diameters have become more available, B-TACE has been widely used, primarily in Japan (Figure 1). In performing B-TACE, evaluation of the collateral circulation of the tumor is essential, as a good response rate is obtained if the catheter tip pressure is equal to 64 mmHg or less; the collateral circulation pressure increases above that in many cases[31].

PLATINUM COMPLEX AGENTS

In advanced HCC treatment, it is critical that TACE functions as an efficient drug delivery system. Platinum complex agents are anti-cancer agents that cause DNA damage. Unlike anthracyclines, which are excreted from the bile, platinum complex agents are not metabolized by P450, and are excreted primarily in the urine. Thus, platinum complex agents are considered to be advantageous for patients with liver cirrhosis. Cisplatin, a first-generation platinum complex, and miriplatin, a third-generation platinum complex, are both used in the treatment of HCC[32-39].

Kawamura *et al*[19] reported that a 19.6% response rate was obtained by switching the anti-cancer agent used in TACE to a platinum complex agent in cases in which the tumor number or size increased despite administration of more than one TACE treatment. Use of a platinum complex agent also resulted in a survival benefit in responders[19]. Furthermore, Maeda *et al*[40] also reported the efficacy of TACE using cisplatin in patients with HCC that had not responded to TACE using epirubicin, with a response rate of 27.5%[40].

However, cisplatin is associated with serious side ef-

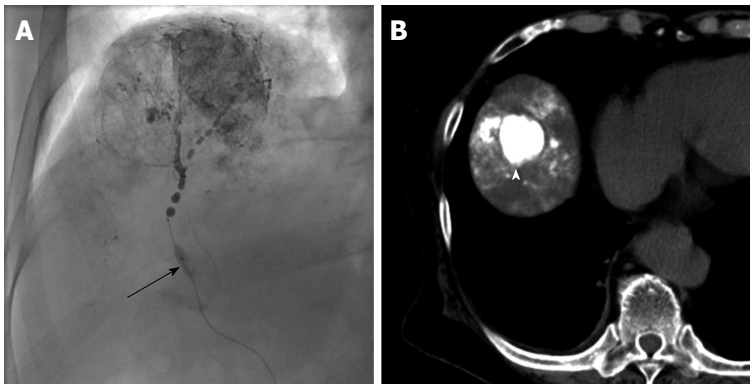

Figure 1 A patient with unresectable hepatocellular carcinoma who received balloon-occluded transcatheter arterial chemoembolization with miriplatin. A: Micro balloon catheter was inserted into A8. Feeding artery was occluded using micro balloon (arrow). Miriplatin/lipiodol suspension and 1-mm gelatin sponge particles were administrated slowly under balloon occlusion; B: Treated lesion showed a dense accumulation of lipiodol (arrowhead).

fects, including renal failure and anaphylaxis. Kawaoka *et al*[33,41] reported that anaphylaxis occurs more frequently during performance of three or more than three TACE procedure with cisplatin. In recent years, miriplatin has been administered as a third-generation platinum complex that has been developed for hepatic arterial infusion therapy particularly for HCC. Miriplatin {cis-[(1R,2R)-1,2-cyclohexanediamine-N,N')bis(myristato)]-platinum(Ⅱ)monohydrate; Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan} is a novel lipophilic cisplatin derivative that can be suspended in lipiodol. Miriplatin/lipiodol suspension is a stable colloidal emulsion that is deposited within HCC tumors, where active derivatives of miriplatin are gradually released. Also, in a cisplatin-resistant rat hepatoma cell line model, miriplatin did not show cross-resistance with cisplatin[37].

Despite clinical expectations, it is difficult to obtain adequate deposition of miriplatin in HCC, and local recurrences, particularly intra-tumoral recurrences, frequently develop using selective TACE[42]. This may be due to the higher viscosity of miriplatin, since it is suspended in lipiodol; miriplatin may be retained within the artery, so a sufficient amount of the drug does not reach the tumor through narrow blood vessels.

Seko *et al*[43] reported that reduction of the miriplatin/lipiodol suspension viscosity resistance can be obtained by warming it to 40 ℃. Compared to ordinary (room temperature) miriplatin treatment, which has a response rate of 44.3%, efficiency is improved to 70.1% for warmed miriplatin treatment[43]. Kora *et al*[44] also reported similar treatment outcomes for warmed miriplatin.

Miriplatin is known to have less serious side effects and a lower incidence of renal failure compared to other platinum complex agents. Thus, miriplatin is considered to be suitable for repeat treatments, patients with complications, and elderly patients[45,46].

DRUG-ELUTING BEAD

In recent years, beginning in western countries, permanent spherical embolic material (*i.e.*, beads) have been used in TACE with the aim of more efficient drug delivery[47-49]. Unlike conventional gelatin sponges, the particle size of this material is uniform. Prediction of the level of embolism is straightforward, and a sustained embolic effect can be obtained. It is also possible to impregnate anti-cancer drugs into the beads, and the anti-tumor effect is improved due to the slow release of anti-cancer agents into the tumor.

Two formulations of drug-eluting beads (DEB) are available in Japan: Hepasphere[50] and DC Bead[51] are widely used and each have unique features. DC Bead is a raw material derived from polyvinyl alcohol that is capable of impregnation of positively-charged drugs (*e.g.*, epirubicin, doxorubicin, or irinotecan). Its size is slightly decreased, and its hardness is increased by impregnation of anti-cancer drugs. Meanwhile, Hepasphere is a raw material derived from a polymer with high water absorption and can thus be impregnated with water-soluble anti-cancer agents. The size of Hepasphere increases following impregnation, it expands to about four times its size in the blood, and the resulting embolus is highly flexible and molds to the shape of the target vessel.

In a randomized controlled trial (PRECISION V) that compared TACE using lipiodol (cTACE) to TACE using DC Bead, the complete response rate, objective response rate, and disease control rate were superior in the DC Bead group compared to the cTACE group, although these differences were not statistically significant. In addition, response rates were significantly higher in certain sub-groups, such as in patients with a Child-Pugh score B and in those with HCC in bilateral lobes[52]. Vogl *et al*[53] also reported that the incidence of decreased left heart ejection fraction, post-embolization liver enzyme elevation, and hepatobiliary system adverse events were lower in the DC Bead group compared to the cTACE group[53]. Sacco *et al*[54] reported similar results from a randomized controlled trial of DEB-TACE *vs* cTACE for unresectable HCC: post-treatment elevation of alanine aminotransferase was frequently observed in the cTACE group. However, time to progression and survival did not significantly differ between the two groups: the cumula-

tive 2-year survival rates were 86.8% in the DEB-TACE group and 83.6% in the cTACE group[54].

For TACE with epirubicin-eluting Hepasphere, Seki *et al*[55] reported a 1-mo response rate of 56.3% and a 6-mo response rate of 52.6%, using response rates as defined by the EASL criteria[55]. For the treatment of HCCs that became refractory to TACE with epirubicin-eluting Hepasphere, changing the impregnated anti-cancer drug to cisplatin resulted in a response at 6 mo in 40% of patients[56].

Several retrospective studies showed the safety and efficacy in DEB-TACE group were significantly higher than in cTACE group (Table 1)[57-59].

However, clear evidence of DEB-TACE superiority compared to cTACE has not been established to date.

LIMITATIONS

There are limitations in this review. First, this is not a systemic review. Therefore, this article may have the potential biases of the authors. Second, we mostly described Japanese history of TACE for HCC in this review.

CONCLUSION

Improvement of the therapeutic effects of TACE treatment for HCC has been obtained by progression in techniques, drugs, and therapeutic equipment. In a variety of TACE treatments, selecting the anti-cancer agents, treatment methods and equipment for the best therapeutic effect is becoming more important. In the future, it is necessary to clarify the optimal treatment choices for each HCC patient.

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TOPIC HIGHLIGHT

WJH 6th Anniversary Special Issues (3): Hepatitis B virus

How did hepatitis B virus effect the host genome in the last decade?

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Abstract

The principal reason of chronic liver disease, cirrhosis and hepatocellular carcinoma is chronic viral hepatitis all over the world. Hepatitis B virus (HBV) has some mutagenic effects on the host genome. HBV may be exhibiting these mutagenic effects through integrating into the host genome, through its viral proteins or through some epigenetic mechanisms related with HBV proteins. This review aims to summarize the molecular mechanisms used by HBV for effecting host genome determined in the last decade. The focus will be on the effects of integration, HBV proteins, especially HBV X protein and epigenetic mechanisms on the host genome. These interactions between HBV and the host genome also forms the underlying mechanisms of the evolution of hepatocellular carcinoma.

Key words: Hepatitis B virus; Host genome; Integration; Hepatitis B virus proteins; Epigenetic

Core tip: Hepatitis B virus (HBV) has some mutagenic effects on the host genome. This review aims to summarize the molecular mechanisms used by HBV for effecting host genome determined in the last decade.

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INTRODUCTION

There are more than 350 million people who are infected by hepatitis B virus (HBV) throughout the world[1]. HBV is the main cause of some liver illnesses, such as chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC)[2]. The World Health Organization classifies HBV in "group 1" as the consequential oncogenic factor after tobacco smoking. It has been estimated that due to late diagnosis and limited treatment options, after lung and stomach cancer, HCC is third prominent cause of cancer related death and nearly 53% of HCC cases has a connection with HBV. The incidence of HCC changes according to geographical conditions. Chronic HBV infection is the most important risk factor for HCC in the world. Other risk factors such as chronic hepatitis C virus (HCV), hepatitis D virus or human immunodeficiency virus infection, aflotoxin B_1 exposure, metabolic factors as obesity and diabetes and alcohol abuse increase the comparative endanger for tumor progression when coexist with HBV infection. Moreover, demographic factors such as Asian or African ancestry, male sex or advanced age are the synergistic effects that have been reported to raise the possibility of HCC in chronic HBV-infected individuals[3-7].

Even though many pathways and factors contributing to HCC development have been identified, many features of hepatocellular carcinogenesis and direct role of viral factors are difficult to define[3]. However, HBV infection is the main risk factor for HCC development. Not only in HCC, but also in chronic HBV patients and chronic HBV carriers some mutagenic effects of HBV on somatic cells

are detected. For example in our study, we proofed the genotoxic effects of HBV on peripheral blood lymphocytes of chronic HBV patients and chronic HBV carriers[8]. Ucur *et al*[9] showed the increased sister chromatid exchange frequency and low mitotoic index; Bolukbas *et al*[10] and Grossi *et al*[11] demonstrated DNA damage using the alkaline comet assay, in peripheral blood lymphocytes. HBV may harm to host DNA in many ways. But simply it can be categorized in at least 3 different mechanisms: First, the viral DNA integration in the host genome can induce chromosome instability, although HBV usually persists as an episome and the integrate genomes are dead and can no longer drive HBV replication. Second, insertional mutations of *HBV* are known to activate many genes and promote genetic alterations in the host genome. The third mechanism is based on viral proteins and gene products of sporadically truncated *HBV* genes from integrated HBV DNA[3,5]. While a variety of manuscripts have been published about these mechanisms separately, here a short review describing effects of HBV on host genome in the last decade is given. The focus of this review will be on integration, proteins and epigenetic mechanisms of HBV.

DIRECT EFFECTS OF INTEGRATION ACTIVATED BY THE HBV DNA ON THE HOST GENOME

Integration is not necessary for the viral replication but it enables viral genomic persistence. Long term chronic inflammation related to continuous cycles of cell death and proliferation increases the amounts of DNA ends in host genomic DNA, thus supporting the viral integration. Cellular topoisomerase I is a crucial factor in the linearization and integration of viral replicative mediators[3,7]. Several kinds of changes in the sequence of HBV genome have been identified, but inverted duplications and the deletions are the most common alterations[12]. Disclosure to oxidative stress or mutagenic agents, loss of DNA repair capacity, high hepatocyte turnover due to inflammation and/or coinfection with other viruses may be the reason of HBV DNA integration[3,7]. In these conditions the genome is more unstable and tend to the devolopment of deletions, single- or double-stranded breaks or rearrangements[12]. HBV integration can induce rearrangements and/or partial deletions at the integration site of the host chromosome[6]. Integration happens by chance in the contex of human genomes and may ocur at the various places of different chromosomes[7]. Translocations, production of fusion transcripts, chromosomal deletions and generalized genomic instability may be caused by these integrations, and alterations probably cause the choosing of hepatocyte clones that have a growth profit[3,12]. Liver carcinogenesis may occur as a result of HBV integration that cause a significant increase in an anti-apoptotic or oncogenic signal[12]. Using a polymerase chain reaction (PCR) based approach (*i.e.*, inverse PCR, Alu PCR, restriction site PCR)[12], it was confirmed that insertion of HBV into cellular genome is an event that happens during HBV infection even after acute self-limiting hepatitis[6]. In 85%-90% of HBV-related HCC, integrated viral DNA has been detected[3,7].

Most of the integration events reported occur near or within common fragile sites (large genomic regions that are liable to deletions, breaks, chromosomal rearrangements and gene amplifications)[12], in genes managing proliferation control, cellular signal transduction cascades, cell viability[6] or Alu sequences and microsatellites (other repetitive human genomic regions) that are liable to instability in carcinogenesis development and growth. Integrated viral DNA into coding regions or cellular regulatory regions of the genome may alter gene expression (cis-activation) or change the structure and function of the produced cellular proteins which possibly cause malignant transformation[3,7]. Insertion of viral DNA may also cause hepatocellular malign transformation *via* the production of mutated viral proteins such as preS/S proteins or truncated X proteins which may trigger signalling cascades in carcinogenesis (trans-activation)[7]. Moreover, HBV genome has enhancer elements that can activate heterologous promoters in a orientation- and position-independent manner[13]. The study of many groups suggest that the HBV enhancers are able to transactivate cellular genes up to 100 kb distant from the integration site[12].

In the late 1970s and early 1980s, the primer investigations defining cellular genomic regions of HBV integrations were carried out[12]. In humans, the recurrent integration of the viral genome into or in the proximity of a host genome has been suggested for HBV in 1987 and in the development of HCC in 2000s. In fact, woodchuck hepatitis virus causes liver cancer by targeting myc oncogenes (*N-myc*, *c-myc*, and *N-myc2*) and some cases of HBV integration into important cellular genes have been informed in human liver tumors (*e.g.*, retinoic acid receptor beta, cyclin A). However, HBV integration regions targeting host genes have not been defined in the past[13]. But in the last decade, there have been many investigations focusing on the integration sites of HBV and insertional mutagenesis seen in HCC.

Paterlini-Bréchot *et al*[13] have isolated nine DNA integration regions from nine hepatocellular carcinomas, demonstrating that the viral genome make mutations in the important regulatory host genes by using HBV-Alu PCR. These genes have a role in cell proliferation and/or differantiation and/or survival: interleukin (IL)-1R-associated kinase 2 gene, neurotropic tyrosin receptor kinase 2 gene, inositol 1,4,5-triphosphate receptor type 2 (*IP3R2*) gene, p42 mitogen-activated protein kinase 1 (*p42MAPK1*) gene, alpha 2,3 sialytransferase gene, *IP3R1* gene, EMX2-like gene, human telomerase reverse transcriptase (*hTERT*) gene and thyroid hormone uncoupling protein gene (Table 1). Different genes, which were located at the HBV DNA integration region, have a specific key cellular function or share common cell signalling cascades. Also, they found that HBV targets both the telomerase gene

Table 1 Genes targeted by hepatitis B virus DNA integration

Gene	Description	Ref.
NTRK2	Neurotropic tyrosin receptor kinase 2	Paterlini-Bréchot *et al*[13]
IRAK2	Interleukin-1R-associated kinase 2	Paterlini-Bréchot *et al*[13]
MAPK1	Mitogen-activated protein kinase 1	Paterlini-Bréchot *et al*[13]
IP3R2	İnositol 1,4,5-triphosphate receptor type 2	Paterlini-Bréchot *et al*[13]
IP3R1	İnositol 1,4,5-triphosphate receptor type 1	Paterlini-Bréchot *et al*[13]
ST3GAL VI	Alpha 2,3 sialytransferase	Paterlini-Bréchot *et al*[13]
TRUP	Thyroid hormone uncoupling protein	Paterlini-Bréchot *et al*[13]
EMX2-like	Empty spiracles 2-like	Paterlini-Bréchot *et al*[13]
hTERT	Human telomerase reverse transcriptase	Paterlini-Bréchot *et al*[13]
WBSCR1	Williams-Beuren syndrome critical region 1	Kimbi *et al*[14]
AXIN1	Axis inhibitor 1	Minami *et al*[16]
BBX	Bobby sox	Minami *et al*[16]
CTNND2	Catenin delta-2	Minami *et al*[16]
EYA3	Eyes absent 3	Minami *et al*[16]
ODZ2	Odd Oz 2	Minami *et al*[16]
TERT	Telomerase reverse transcriptase	Sung *et al*[18]
MLL4	Mixed-lineage leukemia 4	Sung *et al*[18]
CCNE1	Cyclin E 1	Sung *et al*[18]
FN1	Fibronectin 1	Ding *et al*[21]
SMAD5	SMAD family member 5	Ding *et al*[21]
PHACTR4	Phosphatase and actin regulator 4	Ding *et al*[21]

and *IP3R* gene in two different tumors. This data shows viral integration may target some preferential regions, especially *hTERT* gene.

Kimbi *et al*[14] amplified HBV and chromosomal DNA from the sera of five patients with uncomplicated acute hepatitis B and one with fulminant disease. In one patient with uncomplicated disease, HBV DNA was integrated into host chromosome 7q11.23 in the Williams-Beuren syndrome critical region 1 gene (Table 1). This gene contains a high abundance of Alu repeats, repetitive elements that have shown to be the preferred sites for recombination and for HBV DNA insertion. Moreover, the integrant is within the region commonly deleted in patients with Williams-Beuren syndrome, giving rise to loss of heterozygosity. The investigators also mentioned that clonal expansion of an integrant is required for tumor formation and early integration of HBV DNA might have a role in HBV-induced hepatocarcinogenesis. It is because the integrated viral DNA in early infection is in accordance with the observation, that importation of linear DNA into the nucleus is necessary for insertion of viral DNA into chromosomal DNA, and that such importation is known to occur during the initiation of infection. This data was also examined by Murakami *et al*[15] in the study of detecting the possible persistence of integrated genomes in peripheral blood mononuclear cells (PBMCs) and the exact position of the viral genome, after the clearence of serum HBV surface antigen (HBsAg). Their results showed that HBV genome integrates early during acute viral infections and persists in an integrated form in PBMCs. Another study providing the proof of HBV integration at an early stage of chronic infection in hepatocytes was carried out by Minami *et al*[16]. They examined virus-cellular gene junctions in chronic hepatitis tissues without HCC and by analysing six patients 42 independent viral-host junctions have been obtained and chromosomal locations for 20 of the 42 junctions have been shown. Each integration evidently influenced a single clone in six clones. Among these six genes, axis inhibitor 1 is believed to function as a tumor suppressor; eyes absent 3, homolog of odd Oz 2 and homolog of bobby sox are human homolog of drosophila genes that are critical for organ development, but their roles are still mysterious; catenin delta-2 is an oncogene (Table 1). Moreover, in contrast to the previous claim that HBV integration happens randomly, this study suggests that the integration of HBV into chromosome 3 is preferential.

Mixed-lineage leukemia (*MLL*) 2 and 4 genes are also suggested as preferential targets for HBV DNA integration[2,17]. In addition to TERT and cyclin E 1 genes, Sung *et al*[18] showed integration at *MLL4* gene as previous studies (Table 1). MLL4 locates on chromosome 19q13.1 where an amplification or a frequent rearrangement has been shown in solid tumors. After integration, site specific expression such as HBV X (HBx)/MLL4 proteins and chimeric HBx/MLL4 transcripts proposing an insertional mutagenesis that could functionally have a connection with liver carcinogenesis[2].

New accesses have been improved to identify unique integration sites in high-throughput manner using the next generation sequencing (NGS) technologies, which prevented biased identification and preferential amplification of unique integration sites. In the researches of Sung *et al*[18], Fujimoto *et al*[19] and Jiang *et al*[20] they used complete genomic sequencing to identify genome wide HBV integration by the advantages in NGS technologies. Considering that, whole genome sequencing is overpriced for sequencing wide amounts of specimen, Ding *et al*[21] have improved an optional method for deep sequencing and amplification that joins ligation mediated PCR to Illumina's paired-end adapters. This effective and cheaper method have been called massive anchored parallel se-

quencing (MAPS) method. By this method, in addition to two familiar recurrent target genes, fibronectin 1 and TERT1, they identified novel target genes for HBV integration such as actin regulator 4, SMAD family member 5 and phosphatase (Table 1). They also found that HBV integration prefered chromosome 17 and mostly integrated into human transcriptional sites.

Occult HBV infections (OHBI) have the presence of HBV DNA but are short of available serum HBsAg. Occult infection mechanism is still mysterious, however, many acceptable pathways, such as maintenance in PBMCs and integration into human genomic regions exist. Bhargava *et al*[22] planned to research the molecular pathways lying beneath the DNA damage response activated as a result of OHBI in host cells in their investigation. They found that OHBI causes DNA damage in peripheral blood lymphocytes. It was also found that there was a strong relationship between OHBI and oxidative stress. On the other hand, Pollicino *et al*[23] reported a case of 43 years old man seronegative for HBV and HCV infections and positive for HFE-haemochromatosis, who developed HCC in the lack of severe liver damage. In this study they tried to evaluate the occult HBV infection. HBV-Alu PCR showed HBV integration. This integrant was placed upstream of the partitioning-defective-6-homolog-gamma gene (*PARD6G*) and this gene had overexpression in tumor tissues when we compare it to non-tumor liver tissues. Being a target of transforming growth factor-beta in the tumor invasion and metastasis, PARD6G is included in the polarized migration of cells, establishment of cell polarization. These two studies show that OHBI lead to deregulation of gene expression and may alter the oncogenic pathways.

HBV integration effectively surveys the human genome, exerting insertional mutation pressure, and thus may expand the oncogenic opportunities for patients infected by HBV. The most dominant HBV integration sites occur *MLL* and *hTERT* genes. Moreover, there are many candidate genes such as 60S ribosomal protein genes, platelet-derived growth factor receptor, calcium signalling related genes[20,24]. Bok *et al*[25] proposed 3 different models for gene activation in HBV DNA integration on chromosome 11q13 in the SNU cell line: (1) viral integration induces genetic changes and activation of gene expression at the integration site without gene amplification; (2) viral DNA induces gene amplification, causing overexpression during integration and rearrangement; and (3) gene activation is related to gene amplification, regardless of viral integration. These models might also be available for all other gene activation mechanisms in HBV DNA integration. The target sites and integration mechanisms will give information for key genes and pathways included in development of not only HBV and but also non-HBV-induced cancers.

EFFECTS OF HBV PROTEINS ON THE HOST GENOME

Several studies have reported about the procarcinogenic effects of HBV proteins or their randomly truncated transcripts after integration. This part will focus on the effects of HBV proteins, especially X protein, on the host genome in the last decade.

HBx protein

HBx, is a X open reading frame encoded small polypeptide of 154 amino acids, usually produced at very limited amounts during chronic and acute HBV infection. HBx can be found in the cytoplasm of infected hepatocytes and at low level in the nucleus. A variety of HBx functions are still enigmatic[3,6].

The clinical importance of HBx starts with the integration of HBV DNA into the chronic HBV carriers' hepotocytes genome. *X* gene is generally preserved in the integrants, and HBx is frequently seen in malignant hepatocytes of chronic HBV carriers[26]. The integrated HBx often have rearranged forms and may show many deletions, truncation with fusion to cellular DNA or point mutations[7]. One significant information derived from the researches of HBV integration was that 3'-end *X* gene was frequently deleted in HCC cells, and this causes the COOH-terminal truncated HBx protein. This protein, rather than the full length HBx, is needed and adequate to cause HCC[27]. So as to understand the relation between HBV integration and HCC development, Wang *et al*[28] isolated and characterized integrated HBV in 14 primer cases of HCC. The findings showed that C-terminal X protein caused by 3'-deleted *X* gene was observed in 10 samples as a result of HBV integration. These deletions lead to the losses of transcription factor Sp1 binding site, p53-dependent transcriptional repression binding site, and growth-suppressive effect domain, causing cell transformation and proliferation. This result proposes that 3'-deleted *X* gene may have a significant role in the HCC development.

HBx has some controversal effects like pro-proliferative effects and induction of cell cycle arrest or prevention and initiation of apoptosis[3,6]. HBx effects the expression of several genes that are included in signal transduction pathways, metastasis, transcriptional regulation, immune response, metabolism, control of the cell cycle, proliferation and the apoptosis[6,26].

HBx changes expression of cellular gene by triggering cytoplasmic signal transduction pathways [*e.g.*, ras, nuclear factor kappa B (NF-κB), src, activator protein-1 (AP-1), Jak/STAT, PI3K/Akt, Wnt] and by binding to nuclear transcription factors [*e.g.*, activating transcription factor 2 (ATF-2), cAMP responsive element-binding protein (CREB), Oct-1, basal transcription factors], and they both help cell growth and survival[1]. HBx localizations (cytoplasm and nucleus) are associated with different functions. HBx, placed in the nucleus, is proposed to interfere directly with transcription factors or to use a function like a transcription factor. A direct relationship between ATF-2 and CREB concluding in their raised DNA binding affinity[6]. HBx placed in the cytoplasm, where it interferes with and stimulates protein kinases, including IKK, protein kinase C, Jak/STAT, PI3K, pro-

tein kinaseB/Akt, and stres activated protein kinase/Jun N-terminal kinase[26].

HBx is an activator of transcription factor NFκB. HBx stimulated NF-κB promotes liver cells to survive against Fas-mediated apoptosis[26]. However, Zhang *et al*[29] showed another function of NF-κB in their study. Calpain small subunit 1 (Capn4) is included in the HCC metastasis and upregulated in the tissues of HCC. They supposed that HBx might assist migration of hepatoma cell by Capn4. Their results revealed that HBx could upregulate the Capn4 expression at the mRNA and protein levels, and increase Capn4 promoter activity. Interestingly, they found that the inhibition of NF-κB could attenuate the upregulatin of Capn4. Thus, they concluded that HBx upregulate Capn4 through NF-κB/65 to promote migration of hepatoma cells. In another study, Zhou *et al*[30] showed the migration of leukocytes in a NF-κB related pathway. Interferon-γ inducible protein 10 (IP-10) involves in cellular immune damage and inflammatory cell recruitment during virus infection. In their study, Zhou *et al*[30] demonstrated that HBx increases IP-10 expression and the effect of HBx on IP-10 induction is blocked by the addition of the NF-κB inhibitor. Consequently, they reported that HBx affects NF-κB pathway which leads to IP-10 promoter transactivation and then increases leukocyte migration, thus causes immune pathological injury of liver.

HBx interacts with transcription machinery, in addition, there is evidence that HBx involves in the stages of apoptosis. HBx affects the regulation of apoptosis through its role on survivin, caspases, and mitochondria. It has been shown that HBx blocks caspase 3 activity[26]. The elevation of cytosolic calcium signals seems to play a possible role in stimulation of cell proliferation and transcription pathways. Direct interaction of HBx with endoplasmic reticulum (ER) and mitochondria as well as integration events of the X open reading frame were reported to alter intracellular calcium homeostasis[3]. Having a role in caspase-3-dependent pathway, HBx pertubs homeostasis of intracellular Ca^{2+}. This is an important effect in the control of HBx-related apoptosis. HBx possibly have a contact with Bcl-2 during hepatic apoptosis. Proapoptotic activity of HBx bypasses or gets over the Bcl-2 inhibitory effect[26]. Survivin is a apoptosis preventer protein and is overexpressed in a majority of human tumors. HBx can upregulate the expression of survivin in hepatic tumor cells[31]. Moreover, several factors, for example transforming growth factor (TGF)-β, induce PI3K and its downstream target, protein kinase B/Akt, to inhibit apoptosis. HBx downregulates TGF-β-induced apoptosis in hepatocytes by stimulating activity of PI3K[26].

UV-damaged DNA binding protein 1 (DDB1) works as an E3 ubiquitin ligase complex subunit[32] and has been shown to help cell cycle regulation and DNA repair[26,32]. HBx binds to DDB1 and by this way replication of HBV genome is stimulated in the nuclear compartment of cells. HBx needs this nuclear interaction with DDB1 also for interfering with cell viability. It has been demonstrated that HBx triggers lagging chromosomes during mitosis, which then causes arrangement of abnormal mitotic spindles and cells with multinucleus. These formations demand the binding of HBx to DDB1. Thus, this binding may induce genetic instability in regenerating hepatocytes; therefore causes to HCC development[32].

The human *p53* genes' transcriptional repression is caused by HBx and it has capacity to bind to the p53. HBx C-terminal region is needed for sustaining the p53 in the cytoplasm and blocking the p53-mediated apoptosis. However, a tremendous excess of p53 is found when it is compared to HBx in the hepatocytes[6,26]. It seems that the anti and proapoptotic effects of HBx depends on the status of hepatocyte differantiation[3].

Telomerase which adds repetitive DNA sequences to the telomeres, the ends of the chromosomes, is a ribonucleoprotein. By this way it prevents telomere shortening and cell death[33]. Telomerase activation has been implied in immortalization and malignant transformation of cells in vitro and is a vital step in tumor and cellular senescence[3,26]. Although telomerase is a complex comprising a catalytic subunit (hTERT) and an RNA component (hTER), hTERT is the crucial factor of telomerase activity in human cells[34]. High levels of hTERT mRNA in HCC of several grades were found by researchers and they originate in cells which have gone through the molecular changes of the first steps of hepatocarcinogenesis[33]. It has shown that HBx gene can up-regulate the transcriptional expression of hTERT mRNA[35]. In the study of Su *et al*[36], it was found that by transcriptionally repressing its promoter, HBx down-regulated the human telomerase expression. They evaluated human telomerase promoter and identified myc-associated zing finger protein (MAZ) as a transcriptional repressor of the promoter in order to find out the molecular mechanism. It was found that the physical association of HBx with MAZ, supresses human telomerase by enhancing MAZ binding to its consensus sequence in the promoter. In this situation, HBx acts as a transcriptional corepressor.

HBx can also lead to both stabilization of hypoxia-inducible factor-1 and overexpression of vascular endothelial growth factor gene. It seems HBx causes carcinogenesis *via* the alteration of angiogenic pathways[7].

In addition to being involved in angiogenic pathways, HBx can contribute to tumor cell invasion by the way that includes the up-regulation of heat shock protein 90 alpha (HSP90alpha). HSP90alpha isoform is an ATP-dependent molecular chaperone which sustains the effective structure of client oncoproteins in tumor cells. Li *et al*[37] showed that HBx triggers expression of Hsp90alpha at the transcriptional level. HBx is directly included in the HSP90alpha transcriptional activation mediated by c-myc. Moreover, by activation of Ras/Raf/ERK1/2 cascades HBx triggers c-Myc expression, which causes c-Myc-mediated HSP90alpha promoter activation first and then HSP90alpha expression up-regulation. HSP70 and HSP60 have also been shown as a HBx cellular targets[26].

In conclusion, the HBx protein is a multifunctional and very important viral protein in the initiation of he-

patocellular transformation and cell survival during the HBV infection. It interacts with alot of molecules and involves in many cellular pathways. Variations in the role of HBx in hepatocarcinogenesis may be due to hepatocyte differantiation, different functions of truncated X protein and amount of expressed HBx. More information about HBx might highlight many mechanisms involved in HCC and give insights and tools for therapeutic means.

Surface proteins

Large hepatitis B (LHBs) and Middle hepatitis B (MHBs) virus surface proteins are encoded by the preS1/preS2 sequences of HBV. Experimental data revealed that HBV *preS/S* genes truncated at the 3' end and integrated into the cellular genes have a transcriptional activator function and encode proteins that accumulate in the ER. These *preS/S*^{t} genes encoded for C-terminally truncated surface proteins (MHBS^{t}) exhibit regulatory functions, such as the transactivation of host genes involving c-Ha-ras, c-myc, and c-fos oncogenes and the precise activation of the c-Raf-1/MEK/Erk2 signaling essential for AP-1 and NF-κB activation. Described processes result in increased hepatocyte proliferation[3,7].

HBV surface proteins accumulate in ER. Accumulation of proteins in ER is known to trigger apoptosis in the presence of prolonged and severe stress due to an induction of an oxidative stres[3]. PreS-mutant LHBs might also accumulate in ER and with induction of genomic instability and oxidative DNA damage, they become the reason of stress-signalling pathways. This also causes defective DNA damage response and repair in liver cells expressing HBV surface antigen[38]. Moreover, centrosome multiplication and the overexpression of both cyclin A and cyclooxygenase 2 might be caused by pre-S2 mutant proteins, therefore inducing cell cycle progression, chromosome instability and proliferation of hepatocytes in HBV related HCC[3,7]. Churin *et al*[39] also demonstrated that the expression of HBV surface proteins in the liver of transgenic mice induces phosphorylation of eukaryotic initiation factor 2α (eIF2α) and protein kinase like ER kinase activation. eIF2α phosphorylation resulted in activation of ER stress markers and a proapoptotic protein. Morover, by searching on two groups of mice with different genetic backround, they showed that hepatic HBV surface protein expression induced tumor development and fibrosis depends on host genetic backround.

HBV surface proteins may also alter the expression of some host genes with known functions as proved in the research of Rao *et al*[40]. It has shown that HBV-encoded small surface protein (SHBs) has an influence on hepatic cell expression of host genes related to fatty acid synthesis and decomposition.

Core protein

The HBV core protein (HBc) is a 21-22 kDa protein that affects the human immune response. HBV genome encode for the core protein to form the viral capsid[3]. HBc may interact with the human genome in HBV infected liver cells. HBc was demonstrated to suppress the the human carcinogenesis related genes expression, p53 and interferon beta; inhibits tumor necrosis factor-related apoptosis-inducing ligand induced apoptosis in hepatocytes *via* blocking gene expression of the pathway-associated death receptor[41].

However, it is not known how HBc interacts with human genome. Guo *et al*[41] examined the distribution of HBc binding to promoters in the human genome and assessed its effects on the associated genes' expression. It was shown that HBc antibody immunoprecipitated nearly 3100 human gene promoters. The high CpG density promoters were found most commonly among this set of gene promoters. HBc is able to bind to 41 gene promoters of the WNT/β-catenin signalling pathways and 64 gene promoters of the MAPK pathways. These are two most important pathways involved in HBV-related HCC. Moreover, HBc is able to increase host NF-κB DNA-binding ability, thus in order to enhance or inhibit other host nuclear proteins' transcriptional activator functions, HBc may bind to and interact with them. As a result, HBc can bind to a great amount of human gene promoters throughout the whole human genome. This key finding may represent one of the pathogenic mechanism of HBV infection.

EPIGENETIC EFFECTS OF HBV ON THE HOST DNA

According to the recent researches virus and host interactions also happen epigenetically. Interactions between epigenetic machinery and the viral proteins may cause changes in the epigenetic landscape of the cell thus causing cancer. Histone modifications and DNA methylation are epigenetic mechanisms which have an important role during HBV replication[42].

Bisulfite sequencing is the gold standart technique to analyze the methylation state of individual cytosines. In order to acquire complete DNA methylomes of double-stranded DNA viruses like HBV, Fernandez *et al*[43] joined the use of model organisms and bisulfite genomic sequencing of multiple clones. Most importantly, they found that DNA methylated *HBVgp2* and *HBVgp4* genes, which respectively code for the S and C viral proteins, have lost their expression. It was also shown in their sequencing data that the vast majority of the HBV genomes kept the *HBVgp3* gene coding for the X protein in an unmethylated condition. Interestingly, HBX protein might regulate DNA methyltransferase (DNMT) activity.

The expression of DNMTs, which catalyze the addition of a methyl group to the cytosine ring of the 5'-CpG dinucleotide, is often raised in livers infected with HBV and also in HCC[7,44]. The roles of the major methyltransferases have the following roles: DNMT1 adds methyl groups to the hemimethylated CpG dinucleotides and has an important function in the supply of methylation during cell division; DNMT2 lacks DNA methyltransferase capabilities and seems to take part in adding methyl

groups to the structural RNA; DNMT3a and DNMT3b is able to methylate not only unmethylated, but also hemi-methylated CpG dinucleotides[45]. Several studies have reported that both HCC and HBV-infected cells exhibit increased levels of DNMT1, DNMT3a and DNMT3b and aberrant DNA methylation[44].

It was reported that the overexpression of HBx protein induces transcription of *DNMT1*, *DNMT3a* and *DNMT3b* genes and directly interacts and activates the *de novo* methyltransferase DNMT3a. It suggests that this viral protein may be responsible for methylator phenotype in HBV related HCC[7,44].

In their study, Vivekanandan *et al*[45] have revealed that HBV infection up-regulates DNMTs, which then causes the methylation of HBV DNA by DNMT3a. This methylation causes the production of pregenomic RNA, viral mRNA, and a decreased production in viral protein. However, in the same cells, the up-regulation of DNMTs also causes methylation of host CpG islands overlapping gene promoters related to carcinoma[45]. It was demonstrated that specific gene promoters are methylated in HBV infected cells such as metallothionein-1F, IGFBP3, SUFU, and TIRAP[44,45]. HBx may also inhibits transcription of E-cadherin[46], $p16^{INK4A}$ and glutathione S-transferase P1 *via* CpG methylation of the regulatory elements[7]. Some immunoregulatory genes active against HBV can also be methylated. For instance, HBV replication is able to cause *de novo* methylation and decrease the IL-4 expression, that favors the virus because HBV replication is repressed by the IL-4 expression[45].

In addition to methylation processes, it has also been shown that HBx associates with components of histone modification machinery, such as HDAC and CBP/p300 HAT. So it has an effect on gene expression[42].

In summary, HBV infection up-regulates DNMTs in hepatocytes. This up-regulation causes methylation of viral DNA, however specific and critic genes in the host genome can also be methylated. It is certain that, these modifications are significant factors in the development of HCC.

CONCLUSION

At present, in terms of the experimental approaches used, such as PCRs, gene clonings, microarrays, immunohistochemical methods, it is possible to explore the effects of HBV on the host genome. In the last decade, the investigations about the pathogenic effects of HBV on the host genome have mostly focused on HCC development. During this development period, a dominant oncogene isn't encoded by HBV genome, however it uses multifactorial pathogenic mechanisms. In addition to direct mechanisms, which are mainly represented by integration of HBV DNA into the cellular genes and by the production of proteins with transforming capacities, indirect mechanisms involving HBV proteins disrupting vital molecular pathways may be seen in HCC development. Although it is thought that HBV DNA integrations are random and there is no specific region of integration, it is not fully incidental but instead seems to be partly optional. Special integration sites change the expression of various components of the cell cycle, signalling, transcription and apoptotic pathways. It is also shown that the formation of truncated HBV proteins, the activation of host genes by integrated HBV enhancer sequences and modifications in the epigenetic machinery of the host cell are important carcinogenic mechanisms in HCCs.

Although the main risk factors for HBV-induced hepatocarcinogenesis are well known, we still lack a deeper understanding of molecular pathways disrupted by HBV and the relationship between key molecules in these pathways. Therefore, molecular understanding of the mechanisms in HBV-related HCC is necessary for defining risks and identifying novel therapeutic approaches.

Cloning and fully characterizing HBV integrations, phage library construction and sequencing are still the gold standards. Recently, several new methods have been developed using NGS technologies avoiding optional amplification and biased identification of unique integration sites. For example, complete genomic sequencing and an efficient, cost effective method, MAPS, have been developed in high-throughput manner. In the future, novel easy and not time consuming experimental approaches and new HCC animal models might be developed to invent promising therapeutic approaches. The specific antiviral new drugs reducing the chance of integration, decreasing or preventing the harmful epigenetic effects and blocking HBV proteins related with HCC development might provide basis for future and might give hope to the patients.

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TOPIC HIGHLIGHT

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Occult hepatitis B virus infection

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Abstract

Occult hepatitis B virus (HBV) infection (OBI) refers to the presence of HBV DNA in the absence of detectable hepatitis B surface antigen. Since OBI was first described in the late 1970s, there has been increasing interest in this topic. The prevalence of OBI varies according to the different endemicity of HBV infection, cohort characteristics, and sensitivity and specificity of the methods used for detection. Although the exact mechanism of OBI has not been proved, intrahepatic persistence of viral covalently closed circular DNA under the host's strong immune suppression of HBV replication and gene expression seems to be a cause. OBI has important clinical significance in several conditions. First, OBI can be transmitted through transfusion, organ transplantation including orthotopic liver transplantation, or hemodialysis. Donor screening before blood transfusion, prophylaxis for high-risk organ transplantation recipients, and dialysis-specific infection-control programs should be considered to reduce the risk of transmission. Second, OBI may reactivate and cause acute hepatitis in immunocompromised patients or those receiving chemotherapy. Close HBV DNA monitoring and timely antiviral treatment can prevent HBV reactivation and consequent clinical deterioration. Third, OBI may contribute to the progression of hepatic fibrosis in patients with chronic liver disease including hepatitis C. Finally, OBI seems to be a risk factor for hepatocellular carcinoma by its direct protooncogenic effect and by indirectly causing persistent hepatic inflammation and fibrosis. However, this needs further investigation. We review published reports in the literature to gain an overview of the status of OBI and emphasize the clinical importance of OBI.

Key words: Occult hepatitis B virus infection; Transmission; Reactivation; Chronic liver disease; Hepatocellular carcinoma

Core tip: Occult hepatitis B virus infection (OBI) is defined by the presence of hepatitis B virus (HBV) DNA without detectable hepatitis B surface antigen. The prevalence of OBI varies according to the different endemicity of HBV infection, cohort characteristics, and detection methods. Increasing research on OBI has been conducted with respect to the following: (1) transmission through transfusion, organ transplantation, or hemodialysis; (2) reactivation in an immunosuppression state; (3) contribution to the progression of chronic liver disease; and (4) increased risk for hepatocellular carcinoma. Further studies are needed to establish its clinical significance and management.

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INTRODUCTION

Hepatitis B virus (HBV) infection is an important global

public issue. Approximately 2 billion people have serologic markers of HBV worldwide, 360 million of whom have chronic HBV infection. The natural course of HBV infection is determined by the interactions between the host and the virus. Chronic HBV infection is characterized by persistent HBV surface antigen (HBsAg) positivity and viremia. In the past, clearance of HBsAg expression in patients with chronic hepatitis B was considered as disease remission and disappearance of viral DNA[1]. However, the "occult" or "silent" form of HBV infection was first reported in the late 1970s in blood donors with anti-hepatitis B core (anti-HBc) antibody without HBsAg who transmitted hepatitis B[1,2]. The meaning of this clinical entity was reviewed in 1998 by a panel of European and US scientists as a part of the serological pattern "anti-HBc" alone, although the term occult was not used at that time[3]. Increasing data showed persistent low levels of HBV DNA in serum and liver tissues after HBsAg clearance was observed during acute self-limited or chronic HBV infection. Demonstration of this clinical entity has brought about the concept of "occult" or "silent" HBV infection, indicating the presence of HBV DNA in the absence of detectable HBsAg[3,4]. Owing to the development of highly sensitive molecular biology techniques, the clinical and virologic features of occult hepatitis B virus infection (OBI) have been revealed, and its clinical significance has been highlighted recently[5-7]. In this paper, we reviewed the status of OBI with respect to its definition, epidemiology, diagnosis, and mechanism. We also focused on the clinical importance of OBI by focusing on 4 processes: transmission, reactivation, contribution to the progression of hepatic fibrosis, and hepatocellular carcinoma (HCC) occurrence, based on results of available reports.

DEFINITION AND CLASSIFICATION

Several definitions of OBI have been suggested. In the 2008 international workshop held in Italy, OBI was defined as the presence of HBV DNA in the liver (with or without HBV DNA in serum) without HBsAg as determined by using the currently available assays[8]. Serum HBV DNA can be either detectable or undetectable, and when detectable, the level of HBV DNA is usually very low (< 200 IU/mL). When serum HBV DNA levels are comparable to those usually detected in cases of overt HBV infection, "false OBI" should be considered. False OBI is usually due to rare infection with *S* gene escape mutants, which produce a modified HBsAg that is not recognized by routinely used detection assays[9,10]. Defining OBI according to the presence of liver HBV DNA expression is the most accurate because HBV DNA in the liver can be detected even when HBV cannot be detected in serum[11]. However, obtaining hepatic HBV DNA is difficult in clinical practice, and assays for the detection of HBV DNA in liver tissue have not been well standardized[7].

Detection assays for HBV DNA in serum have been used with sufficient sensitivity; therefore, OBI is often defined as the presence of serum HBV DNA without detectable HBsAg in clinical practice and in many studies[7,8,12]. Bréchot *et al*[9] proposed to define occult HBV infection as the detection of HBV DNA by using PCR or other amplification assays in HBsAg-negative individuals. Allain[13] also defined OBI as the presence of HBV DNA without detectable HBsAg, with or without anti-HBc or anti-HBs antibody outside the pre-seroconversion window period. This definition of OBI according to the presence of serum HBV DNA is most commonly used in clinical practice.

OBI has also been defined as a serological condition characterized by the presence of isolated hepatitis B core antigen (anti-HBc) in the absence of HBsAg and anti-HBs antibody[3,14]. Detection of anti-HBc antibody, a surrogate marker of OBI, is useful when an HBV DNA test is not available or when intermittent viremia is suspected[8,15]. However, when OBI is defined according to the presence of anti-HBc antibody alone, false anti-HBc positivity and negativity in the detection of OBI should be considered. Not all anti-HBc-positive subjects are HBV DNA-positive. In addition, the absence of anti-HBc antibody does not exclude seronegative OBI. As mentioned earlier, the definition of OBI slightly differs between studies; thus, cautious interpretation should be exercised when comparing study results about OBI.

OBI can be classified into 2 groups, seropositive OBI [anti-HBc and/or anti-hepatitis B surface (anti-HBs) positive] and seronegative OBI (anti-HBc and anti-HBs negative), on the basis of the HBV antibody profile. Seropositive-OBI develops when serum test results for HBsAg become negative after acute hepatitis or when HBsAg is cleared during the course of chronic hepatitis B. In fact, annual HBsAg seroclearance rates are reported to be 0.50%-2.26% per year in chronic hepatitis B patients, and persistent HBV DNA in the liver was detected in some of these patients[16,17]. Seronegative-OBI is caused by primary occult of anti-HBs or anti-HBc from the beginning of the infection because of the mutation or due to progressive loss of anti-HBs[8,18]. Most OBIs are seropositive OBIs, but > 20% of patients with OBI are seronegative for OBI, representing a population negative for all serum markers of HBV infection[19].

EPIDEMIOLOGY

The prevalence of OBI is reported to range from 1% to 95% worldwide. These prevalence rates are influenced by several factors as follows: (1) geographic differences (endemicity); (2) different patient characteristics, including the presence of comorbid diseases such as chronic hepatitis C; and (3) and the different diagnostic techniques used, which have different sensitivity[8,20,21].

The prevalence of OBI differs according to the endemicity of HBV infection. OBI was reported at a higher rate in an HBV endemic area such as East Asia where 41%-90% of the population had prior exposure to

HBV, and less frequently in the low endemic areas such as North America, where only 5%-20% of the population had previous exposure[22]. Several studies on blood donors with similar cohorts have reported a 0.1%-1.05% prevalence of OBI in HBsAg-negative, anti-HBc-positive donors from North America, 0%-1.59% in donors from Europe, and up to 6% in donors from an endemic area[5,23,24].

The prevalence of OBI differs according to the cohort characteristics. OBI is more commonly noted in patients at high risk for parenterally transmitted infections such as hepatitis C virus (HCV) infection or immunosuppression condition such as human immunodeficiency virus (HIV) infection[25]. In particular, OBI prevalence is high in patients with HCV infection, with HBV DNA detected in approximately 33% of patients with HBsAg-negative HCV infection in Italy and > 50% patients in East Asian countries[19,26,27]. OBI prevalence was also high in patients with other chronic liver diseases at 20%-30%[25,28], in hemophilia patients in Japan at 51%[29], and in intravenous drug users at 45%[30]. Among HIV-infected patients in Turkey, 19.1% of subjects had isolated anti-HBc (considered a marker of OBI) compared to only 2.4% in blood-donor controls[31]. The effect of highly active antiretroviral therapy, clusters of differentiation (CD)4 cell count, and HIV RNA load on OBI prevalence is still controversial[24,32,33].

The prevalence of OBI also differs according to the sensitivity of HBV DNA or HBsAg testing. There are various amplification methods for detecting HBV DNA, and the HBV genome target sites are also different. Some commercial assays are more sensitive than others at detecting HBsAg mutants. The type of sample used (liver or serum) or number of samplings can also have some effect on the diagnosis of OBI. Indeed, as serum HBV DNA levels seem to fluctuate in OBI, serial sample is more useful to identify OBI[21].

Since the study population differs significantly based on the above-mentioned factors, prevalence was hard to compare directly among studies. Therefore, caution should be exercised when interpreting the prevalence of OBI in different studies.

MECHANISM

Some researchers insist the lower sensitivity of the HBsAg immunoassay compared to that of polymerase chain reaction (PCR) for the detection of HBV DNA is responsible for development of OBI. However, this difference in the assay sensitivity cannot explain the characteristic lower replication rate of HBV observed in OBI. The precise underlying mechanisms of OBI are not well understood, which could be multifactorial. Both host and viral factors seem to have roles in suppressing viral replication and infection control[5,19].

Host factors

OBI is characterized a low rate of HBV replication *in vivo*; however, occult HBV strains are replication-competent *in vitro*. This suggests that host, rather than viral, factors are more responsible for OBI[34]. Similarly, many clinical observations indicate that OBI reactivation sometimes occurs under immunosuppressive conditions, such as during cancer chemotherapy treatment, HIV infection, or hematopoietic stem cell transplantation[25,35]. This can be explained by a break in the balance between the host's immune system and the virus that occurs during occult infection caused by change in immune system function, resulting in reactivation of OBI. These findings strongly indicate the critical role of the host's immune system in development of OBI.

Other *in vitro* studies showed vigorous antiviral T-cell responses several years after clinical recovery from acute hepatitis B. This suggests persistent synthesis of minute undetectable amounts of virus by HBV covalently closed circular DNA (cccDNA) or other viral transcripts in OBI, maintaining the HBV-specific memory T-cell response[36]. Therefore, these findings also indirectly emphasize the role of the host immune system in the development and maintenance of OBI[25,37,38].

In addition to memory T-cell immune reaction, innate immune system or cytokines such as tumor necrosis factor-α and interferon-γ also have been reported to be associated with OBI[39,40]. Furthermore, epigenetic regulation, including DNA methylation of the HBV genome and posttranslational modification of histones, has been reported to be related to the OBI[41].

Viral factors

Although there is no sufficient evidence, viral factors also seem to have some effect on development of OBI. Several possible mechanisms explaining the low viral replication rate in OBI have been demonstrated. Mutations of the X region of HBV reduce the ability of the X protein to transactivate host cellular proteins that are essential for viral replication, which led to the suppression of replication and expression of HBV DNA, and resulted in negative seropositivity for HBsAg[42]. Escape mutation of the S region was another possible viral factor associated with OBI, which also decreases reactivity in HBsAg detection assays[43]. In addition, a large number of mutations were reported which can reduce HBsAg expression, decrease immune recognition of the virus, and impair HBV packaging. However, cautious interpretation is necessary, as most of these studies lacked a control group or mutations appeared not only in patients with OBI but also those with overt HBV infections. Further studies should be conducted[44].

DIAGNOSIS

Several methods using liver tissue, DNA extracts from liver or blood, or other serologic markers such as anti-HBc IgG have been used to diagnose OBI. The gold standard for OBI diagnosis is the detection of HBV DNA in the DNA extraction from the liver, as cccDNA

persists in the hepatocytes and HBV DNA is sometimes detected in the liver in the absence of HBV DNA in the serum. However, obtaining liver tissue is an invasive procedure; therefore, obtaining hepatic HBV DNA is difficult in clinical practice. In addition, real-time PCR-based assays for serum (or plasma) HBV DNA detection have been used with sufficient sensitivity to detect OBI in many cases; hence, serum HBV DNA assays are widely used to diagnose OBI[8].

DNA should be extracted from samples using the most efficient procedure. A higher rate of HBV DNA detection has been obtained with snap-frozen liver tissue than with paraffin-embedded liver tissue. When a blood sample is used, at least 1 mL of serum should be collected to improve the sensitivity of the test. DNA extracts should be amplified by highly sensitive nested PCR or by a real-time PCR technique that can detect fewer than 10 copies of HBV DNA using the oligonucleotide primers specific for different HBV genomic regions and complementary to highly conserved nucleotide sequences. Appropriate negative and positive controls should be included in each PCR experiment. In addition, periodic testing for HBV DNA will improve diagnosis of OBI especially in high-risk patients, as intermittent viremia can occur in occult HBV infection[8,18,21,22,45].

When highly sensitive HBV DNA testing cannot be performed, anti-HBc could be used as a possible surrogate marker for identifying potential seropositive OBI in cases of blood and organ donation or those receiving immunosuppressive therapy. In this case, seronegative OBI or false-negative anti-HBc in an immunocompromised host should also be considered[45].

CLINICAL SIGNIFICANCE

Transmission of OBI

Transfusion: Although the risk of HBV transmission through blood transfusion has decreased owing to the development of sensitive and specific diagnostic assays, transfusional transmission of HBV still occurs. Transmission of HBV by transfusion occurs in 3 situations: (1) blood from a donor with OBI; (2) blood from patients in the infectious window period of HBV infection; or (3) blood from a donor infected with S-escape mutant HBV infection not detected by the routinely used diagnostic HBsAg assay. The prevalence of OBI in blood donors is variable depending on the geographic area, and is higher in HBV endemic areas[46]. In an Australian study analyzing 2673521 blood donors, the incidence of OBI was approximately 5.55 per 100000 donors compared to the 1.06 per 100000 donors with an acute serologic window period infections[47]. In China, the pooled prevalence of OBI among donors was 0.094%[48]. In Europe, OBIs are detected in 1:2000 to 1:20000 samples donated[45,49-52].

The infectivity of OBI by transfusion is determined not only by the viral load or the volume of plasma but also by the HBV serological status (anti-HBc and/or anti-HBs). The risk of HBV transmission may depend on the presence of anti-HBsAb. Among occult HBV-infected donors, those with high anti-HBs levels (recovered) are unlikely to transmit the infection, whereas those without anti-HBs (anti-HBc only) may transmit the infection[53,54]. However, the infectivity of anti-HBs-containing blood components in immunodeficient or immunosuppressed recipients has not been systematically explored. Considering that immunocompromised hosts represent a substantial proportion of transfusion recipients, caution should be exercised when anti-HBc-positive blood is transfused to immunocompromised recipients, even when anti-HBs positive[45,54].

Nucleic acid testing (NAT) for donor screening detects HBV infection in the window period (before the appearance of HBsAg) as well as OBI, indicating the presence of HBV DNA in the absence of HBsAg. Therefore, the introduction of NAT has further decreased the risk of HBV transmission through blood transfusion. However, cost effectiveness and availability of NAT should be considered before clinical application. Where HBV DNA testing is not available, such as in developing countries, testing for anti-HBc is strongly recommended[8,45,55].

Organ transplantation: OBI in a transplantation donor is important because there is a risk of HBV transmission from an OBI-seropositive donor, and severe HBV reactivation can occur in some of these cases during immunosuppression. As the hepatocytes are the reservoir of HBV cccDNA, the rate of transmission is higher in orthotopic liver transplantation compared to other organ transplantations such as kidney, bone marrow, and heart[25,56]. The transmission of HBV infection from HBsAg negative/anti-HBc positive (considered OBI) donors to recipients were reported at a rate of 17%-94%[57-59]. Because of this high risk of transmission, prophylaxis is recommended to prevent HBV reactivation. Although not directly compared in randomized controlled trials, the combination of antiviral and hepatitis B immunoglobulin seems to be superior to treatment with antiviral or hepatitis B immunoglobulin monotherapies as prophylaxis. Lamivudine is the most widely used antiviral, and studies using newer antivirals such as entecavir, adefovir, and tenofovir are few[57]. It is uncertain whether OBI is transmitted from HBV-seronegative donors.

OBI in liver transplant recipients is also important. The etiology of *de novo* HBV infection after liver transplantation was traced to OBI in both donors and recipients[60].

Hemodialysis: Hemodialysis patients are at increased risk of parenterally transmitted infections because they are in an immunosuppressed state and exposed to invasive procedures, share the same dialysis machine, and receive more transfusions than the general population. The relatively low acceptance and response rates to the HBV vaccine among dialysis patients also likely contributes to OBI transmission in hemodialysis patients[55,61]. The prevalence of OBI in hemodialysis patients varies from 0%

to 54% according to the diagnostic techniques or HBV endemicity[62,63], and several studies suggest that OBI could be a source of viral spread both to other patients and staff within the hemodialysis units[61,62]. Therefore, patients and staff need HBV vaccine boosts to maintain levels of protective antibody to HBsAg (anti-HBs). Strict dialysis-specific infection-control programs, including avoidance of dialyzer reuse and use of dedicated dialysis rooms and machines, should be implemented. Staff for infected patients should be educated on preventive method to limit HBV transmission within dialysis units. Furthermore, regular screening for HBV DNA with sensitive PCR-based assays in all dialysis patients should be considered, and more attention should be given to patients who receive immunosuppressant drugs after renal transplantation[62,64].

Pregnancy and OBI transmission: Kwon *et al*[65] studied the possibility of transmission of OBI to the fetus in 202 healthy pregnant women. Among these, six (3%) women were OBI positive. When cord blood of 4 of these 6 women was evaluated for HBV DNA, all were HBV-DNA negative. This result suggests that vertical transmission through the cord blood is negligible, but this needs to be investigated further[65].

Reactivation

HBV reactivation after systemic chemotherapy was first reported in the mid 1970's, and thereafter, HBV reactivation has been reported not only in HBsAg-positive patients but also in OBI patients[35,66]. Although, reactivation in OBI occurs more rarely than in HBsAg positive patients, HBV reactivation is quite a frequent event in immunocompromised OBI patients when including not only symptomatic hepatitis but also HBsAg re-seroconversion in the reactivation of OBI[66-68]. This finding is clinically important because it can be associated with liver dysfunction, sometimes causing life-threatening fulminant hepatic failure, and often requires interruption of chemotherapy[20,69]. The underlying mechanism of reactivation is thought that chemotherapy induced immunosuppressive state triggers rapid viral replication because of the loss of the immunological control. After immune system reconstitution, cytotoxic T-cell-mediated hepatocyte injury may occur, leading to the development of hepatic inflammation and concomitant hepatic necrosis.

Hematological malignancies, hematopoietic stem cell transplantation, liver transplantation from anti-HBc positive donors, and treatment with anti-CD20 (rituximab) seem to be the factors associated with the highest risk of OBI reactivation[25,70-73]. Other immunosuppressive conditions, including HIV infection, kidney or bone marrow transplantation, systemic chemotherapy, and rheumatologic diseases or inflammatory bowel disease treated with biological agents or high-dose steroids for prolonged treatment, also have been reported as possible causes of viral reactivation in OBI patients[37].

While prophylactic antiviral treatment to prevent reactivation is well established in HBsAg-positive patients undergoing immunosuppressive therapies, its use in OBI patients is debatable[25,70]. In highly endemic areas, 20% of cancer patients have been reported to be HBsAg-negative and anti-HBc positive[74]; thus, prophylactic antiviral use for all OBI patients is unlikely to be cost-effective[68]. On the contrary, delayed treatment with an antiviral may be fatal, and frequent monitoring for reactivation in OBI patients is sometimes difficult. Therefore, use of antivirals is recommended for OBI patients with highest risk of reactivation (previously suggested) regardless of HBV DNA presence and when HBV DNA monitoring is unavailable in routine practice[70,73,75,76]. HBsAg-negative and anti-HBc-positive patients with undetectable or low levels of HBV DNA without highest risk of reactivation should be carefully monitored using alanine aminotransferase (ALT) and HBV DNA levels, with adequate intervals before and during immunosuppressive treatments, and also for several months after stopping treatment. In this case, antiviral treatment should be started as soon as HBV reactivation is detected, before ALT level elevation, since the objective of this strict surveillance is to identify HBV reactivation early before liver injury to prevent acute hepatitis[68,75]. Among antivirals, lamivudine seems to be effective in patients with no or very low serum HBV DNA levels[77]. More potent nucleoside analogues should be chosen when reactivation is confirmed or lamivudine resistance is suggested[73]. Currently, there is no consensus about the optimal duration of preventive antiviral therapy[78]. However, several reports suggest the start of prophylaxis 1-2 wk before the start of immunosuppressive therapy and prolonged antiviral therapy at least 6-12 mo after completion of chemo- or immunotherapy to prevent delayed reactivation of HBV[79,80]. However, further studies should be performed to determine the optimal duration of treatment.

Progression of chronic liver disease

It has been shown that HBV genomes may persist for a long time in the liver, inducing mild necro-inflammation in patients after complete clinical recovery from acute self-limited hepatitis B[81]. An *in vivo* study of a woodchuck model showed similar results; animals that recovered from acute woodchuck hepatitis virus (the rodent HBV-like hepadnavirus) showed lifelong existence of viruses replicating at low levels, inducing mild persistent liver necroinflammation[82]. These results suggest the role of OBI in the progression of chronic liver disease, and there has been much interest in the clinical impact of OBI, both as a mono-infection and as co-infection with HCV, on the course of chronic liver disease.

As HBV and HCV share the same transmission route, OBI is highly prevalent in patients with HCV-related chronic hepatitis. Thus, many cross-sectional studies investigated the influence of OBI on the outcome of chronic hepatitis C. Previous studies showed OBI as a risk factor for more severe liver disease[26,83]; however, the cross-sectional nature of most of these studies could

have biased patient selection. A recent longitudinal Italian cohort study by Squadrito *et al*[84] showed that among chronic hepatitis C patients, patients with OBI had higher risk of progression to cirrhosis, development of HCC, and increased risk of liver-related death compared to OBI-negative patients[84]. Other studies additionally showed the association of ALT level flares with detection of HBV DNA in HCV-OBI co-infected patients, indicating that transient HBV reactivation might be involved in liver injury in these patients[85,86]. A recent meta-analysis of both OBI mono-infection and co-infection with HCV showed that OBI is associated with chronic liver disease, with an overall 8.9-fold increased risk compared to individuals without OBI. Subgroup analysis comparing HCV-positive and -negative subjects showed that HCV-positive as well as HCV-negative patients (cryptogenic liver disease) had increased risk for chronic liver disease[87].

Conclusively, when OBI is present with HCV or with other chronic liver diseases, hepatic inflammation induced by a mild immune response to OBI may accelerate liver injury. In most healthy subjects under immune control, it is not determined yet whether OBI can cause clinically relevant hepatic damage[70,88].

Hepatocellular carcinoma occurrence

HBV infection is known to be one of the most important risk factors in the development of HCC. Although the mechanism by which HBV infection causes HCC is not completely known, HBV causes HCC both indirectly and directly. HBV infection causes hepatic inflammation, regeneration, and fibrosis associated with cirrhosis, which indirectly contribute to HCC development. In a direct pathway, HBV integrates into the host genomes, produces proteins with pro-oncogenic activities, such as X protein and mutant preS-S proteins, and causes genetic and epigenetic alterations that may directly induce hepatocyte transformation[89,90].

Considering that OBI is characterized by intrahepatic persistence of viral cccDNA, OBI can be a risk factor for HCC development in a similar way. In epidemiologic studies, a significantly higher prevalence of OBI was observed in HCV-positive HCC patients than in HCV-positive populations without HCC. Similar results were reported in the HCV-negative patients with cryptogenic liver disease or alcoholic liver disease[37,91-94]. An *in vivo* experimental study also demonstrated that woodchucks, after serological recovery from acute woodchuck hepatitis virus infections, are at high risk of developing HCC even after apparent clearance of the virus[95]. In prospective studies, the cumulative probability of developing HCC was significantly higher among patients with OBI than among HBV DNA-negative patients, both in the presence[96-99] or absence of HCV infection[100]. In addition, a recent meta-analysis demonstrated that OBI increases the risk of HCC in both HCV and non-HCV infected patients[101].

Although these results support the idea that OBI is a risk factor for HCC, caution should be exercised during interpretation[102]. First, as most of the study subjects had chronic liver disease of various etiologies, these results indicate OBI as a co-carcinogen of HCC in addition to other suggested carcinogens such as previous HBV infection, HCV, or alcohol. The role of OBI *per se* in the occurrence of HCC should be further investigated. Second, further studies should be performed to confirm the role of OBI in cryptogenic liver disease. Previous studies have considered heterogeneous definitions of cryptogenic liver disease and non-B non-C liver diseases, (*e.g.*, not differentiating nonalcoholic fatty liver disease and sometimes including alcohol or autoimmune liver disease in cryptogenic liver disease); therefore, it is difficult to interpret the results. Third, several other studies did not find an association between OBI and HCC, and ethnic and epidemiologic differences should be considered in the interpretation of the results[103].

CONCLUSION

OBI, defined as the presence of HBV DNA without detectable HBsAg, has recently gained increasing attention. Although the exact mechanism of OBI has not been determined, OBI can be transmitted, cause reactivation of HBV, and contribute to the development of progressive liver disease and HCC. Thus, physicians should focus on the appropriate management of these patients, and further studies to clarify the clinical significance of OBI are needed.

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TOPIC HIGHLIGHT

WJH 6th Anniversary Special Issues (5): Hepatitis C virus

Functional foods effective for hepatitis C: Identification of oligomeric proanthocyanidin and its action mechanism

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Abstract

Hepatitis C virus (HCV) is a major cause of viral hepatitis and currently infects approximately 170 million people worldwide. An infection by HCV causes high rates of chronic hepatitis (> 75%) and progresses to liver cirrhosis and hepatocellular carcinoma ultimately. HCV can be eliminated by a combination of pegylated α-interferon and the broad-spectrum antiviral drug ribavirin; however, this treatment is still associated with poor efficacy and tolerability and is often accompanied by serious side-effects. While some novel direct-acting antivirals against HCV have been developed recently, high medical costs limit the access to the therapy in cost-sensitive countries. To search for new natural anti-HCV agents, we screened local agricultural products for their suppressive activities against HCV replication using the HCV replicon cell system *in vitro*. We found a potent inhibitor of HCV RNA expression in the extracts of blueberry leaves and then identified oligomeric proanthocyanidin as the active ingredient. Further investigations into the action mechanism of oligomeric proanthocyanidin suggested that it is an inhibitor of heterogeneous nuclear ribonucleoproteins (hnRNPs) such as hnRNP A2/B1. In this review, we presented an overview of functional foods and ingredients efficient for HCV infection, the chemical structural characteristics of oligomeric proanthocyanidin, and its action mechanism.

Key words: Hepatitis C virus; Blueberry leaves; Functional foods; Oligomeric proanthocyanidin; Heterogeneous nuclear ribonucleoproteins

Core tip: An infection by hepatitis C virus (HCV) causes chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. While the combination of pegylated α-interferon and ribavirin is used for the elimination of HCV, a new anti-HCV drug is required due to the poor efficacy and serious side-effects associated with this combination therapy. We searched for new anti-HCV agents from natural products and then identified oligomeric proanthocyanidin from blueberry leaves. Further investigations suggested that several heterogeneous nuclear ribonucleoproteins may be the candidate proteins involved in the proanthocyanidin-mediated inhibition of HCV subgenomic expression. Oligomeric proanthocyanidin isolated from blueberry leaves may have potential usefulness as an anti-HCV compound.

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INTRODUCTION

Hepatitis C virus (HCV) is a major cause of viral hepatitis and currently infects approximately 170 million people worldwide[1,2]. An infection by HCV causes high rates of chronic hepatitis (> 75%) and progresses to liver cirrhosis and hepatocellular carcinoma ultimately[3]. A total of 27% and 25% of individuals that develop liver cirrhosis and hepatocellular carcinoma worldwide, respectively, arise in HCV-infected people[4]. The World Health Organization reported that between 350000 and 500000 people die from HCV-related diseases each year. However, there is no effective vaccine against HCV infection at present.

Currently, the combination of pegylated α-interferon and a broad spectrum antiviral drug, ribavirin, is used as the standard therapy for chronic HCV infection[2,5,6]. However, its option is unfortunately limited by efficacy, tolerability, and significant side-effects. Therefore, it had been required to establish a new therapeutic modality without serious adverse effects. Recently, direct-acting antivirals (DAAs) that inhibit HCV-specific proteins have be clinically investigated[7,8]. For example, boceprevir and telaprevir are new DAAs that were first approved by the United States Food and Drug Administration (FDA) in 2011[9]. DAAs are expected to provide new promising treatment options in hepatitis C patients; however, at present, they face difficulties to disseminate worldwide due to high costs. Therefore, new anti-HCV agents that are safe, economical, and complementary with present therapies, are still required.

Since the development of HCV-related liver cirrhosis and hepatocellular carcinoma requires a prolonged period (20-30 years), the progression of this disease may be influenced by a diet including dairy products. Interest in functional foods and their ingredients as natural resources for cancer prevention and treatment is increasing[10,11]. Eating habits, foods, nutrients contained in them, and other food constituents play important roles on the development of several types of cancer and 35% of cancer deaths are estimated to be possibly related to dietary factors[12]. Polyphenols derived from various fruits and vegetables have recently been suggested to be effective in the prevention of cancer. The South Kyushu region of Japan, including the prefecture of Miyazaki, has been recognized as a high prevalence area of HCV and it emerges as a social issue. Therefore, attempts were made to identify functional food ingredients having suppressive activities against HCV replication as an industry-academia-government collaboration study[13]. By screening of 1700 samples from 283 agricultural products in Miyazaki prefecture, we found that oligomeric proanthocyanidin, a polyphenolic ingredient abundantly contained in the leaves of the blueberry plant, suppressed the expression of HCV subgenomic RNA in an HCV replicon cell system[13].

In this review, we presented an overview of functional foods and ingredients efficient for HCV infection, the chemical structural characteristics of oligomeric proanthocyanidin, and its action mechanism.

HCV LIFE CYCLE AND ANALYTICAL TOOL

HCV belongs to *Hepacivirus* genus of the *Flaviviridae* family and has a positive-sense single stranded RNA of 9.6 kb wrapped with enveloped membrane[14]. After their adsorption on the surface of host cells, HCV particles are internalized into endocytic compartments and viral genomic RNA is then released into the cytoplasm by fusion of the viral envelope and cellular membrane. Genomic RNA serves as mRNA for viral proteins and is translated into a single polyprotein (3011 amino acids), resulting in 4 structural proteins (Core, E1, E2, and p7) and 6 nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) by post-translational processing (Figure 1A). It also serves as a template for viral genome replication. Non-translated regions (NTRs), 5'NTR and 3'NTR, are connected with the HCV polyprotein-coding region, and modulate viral protein synthesis and genome replication. The assembly of these viral components occurs on the endoplasmic reticulum (ER) membrane. Viral proteins and genomic RNA assemble on the cytoplasmic side of the membrane and then progeny virions bud into the ER lumen, followed by their release to the extracellular space. In the life cycle of HCV, each viral protein functions as described below[14]. Core is a highly basic protein that encapsidates HCV genomic RNA. E1 and E2 are glycoproteins integrated into the viral envelope. p7 functions as an ion channel and an antiviral drug, amantadine, is the p7 ion channel blocker[15]. Importantly, several steps of HCV infectious process are coordinated by NS proteins. NS2 and NS3 are a cysteine protease and serine protease, respectively, that play roles in the post-translational processing of viral proteins. NS3 serine protease activity requires NS4A as a cofactor. NS4B and NS5A have been suggested to serve in viral assembly on the ER membrane and NS5B is an RNA-dependent RNA polymerase. Many studies to date have reported that these viral proteins are associated not only with viral replication, but also pathogenicity *via* interactions with various host proteins. The identification of host proteins associated with the HCV life cycle is very important for anti-HCV drugs, and the HCV replicon cell system has contributed significantly to the development of these drugs[16,17]. This system consists of the human hepatocellular carcinoma line Huh-7 in which the transfected luciferase gene connected with HCV subgenomic RNA including the downstream coding regions of NS3 and the expression of HCV subgenomic RNA can be quantified by luciferase activity (Figure 1B). It provides a useful tool for HCV drug development

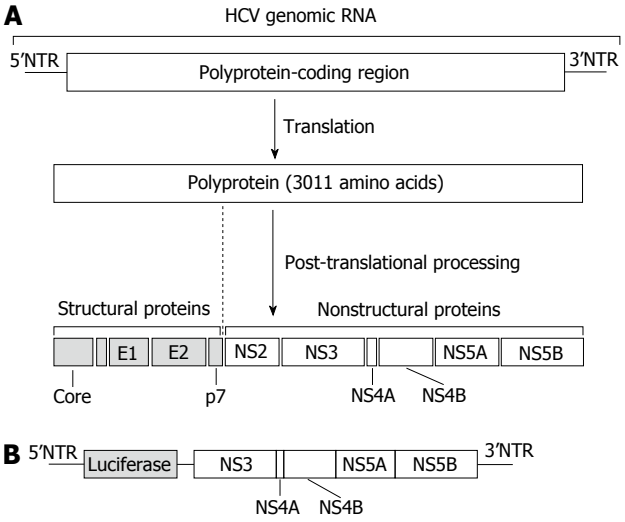

Figure 1 Structure of the hepatitis C virus genome and cell system for anti-hepatitis C virus drug discovery. A: HCV genomic RNA and viral proteins. HCV genomic RNA encodes a single polyprotein of 3011 amino acids. After being translated, the polyprotein is processed into 4 structural proteins (Core, E1, E2, and p7) and 6 non-structural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B). The polyprotein-coding region is flanked by 5' and 3'NTRs. Viral RNA also serves as a template for viral genome replication and both NTRs modulate viral protein synthesis and genome replication; B: The HCV replicon cell system. Huh-7 cells were transfected with the luciferase gene connected with HCV subgenomic RNA including the downstream coding regions of NS3. The expression of HCV subgenomic RNA could be quantified by luciferase activity. HCV: Hepatitis C virus; NTRs: Non-translated regions.

and the elucidation of mechanisms underlying HCV genome replication[17]. We have used this HCV replicon system to screen functional foods with anti-HCV activity.

THERAPEUTIC OPTIONS FOR CHRONIC HCV INFECTION

Currently, the combination of pegylated α-interferon and a broad spectrum antiviral drug, ribavirin, is used as the standard therapy for chronic HCV infection[2,5,6]. However, the HCV genotype is an important determinant of its efficacy and tolerability. Whereas the virological response to this combination therapy is more than 70% for genotypes 2 and 3, it is less than 50% for genotype 1[18-20]. Furthermore, this therapy causes significant side-effects such as thrombocytopenia, flu-like symptoms, fever, rash, anorexia, and thyroid dysfunction. Depression and irritability that are expressed as neuropsychological disorders during therapy impair quality of life universally. Therefore, it had been required to establish a new therapeutic modality without serious adverse effects.

Recently, DAAs that inhibit HCV-specific proteins have been clinically investigated[7,8]. Two DAAs, boceprevir and telaprevir first came to the HCV drug market and were approved by FDA in May 2011. Boceprevir or telaprevir was used as triple therapy with pegylated α-interferon and ribavirin for hepatitis C patients with genotype 1[9]. These DAAs are inhibitors against HCV NS3/4A serine protease and bind covalently with active site of the enzyme[21-23]. The triple therapy using boceprevir or telaprevir significantly increased the rate of sustained virological response (SVR) for naive or previous treated hepatitis C patients with HCV genotype 1[24-29]. After that, next generation DAAs, ABT-450/r, simeprevir, and faldaprevir, which are also NS3/4A protease inhibitors, have been reported to have advantages of their convenience and improved side effects profile[30-32]. Further, daclatasvir and sofosbuvir, which are an NS5A replication complex inhibitor and a nucleotide analogue NS5B polymerase inhibitor, respectively, also increased SVR rate[33-35]. Notably, the combination of these DAAs only was the highly effective treatment for patients with HCV genotype 1[36,37] and it is feasible to treat HCV without interferon and ribavirin.

While patients with hepatitis C can be treated by above mentioned DAAs without significant side-effects, it requires high medical costs and limits access to the therapy in cost-sensitive countries[38]. Of the 20 countries with the high prevalence of HCV, 12 are categorized as low or lower-middle income countries[39]. Therefore, new anti-HCV agents that are safe, economical, and complementary with present therapies, are still required and we focus attention on functional foods and their ingredients.

FUNCTIONAL FOOD INGREDIENTS EFFECTIVE FOR HCV

The development of HCV-related liver cirrhosis and hepatocellular carcinoma requires a prolonged period (20-30 years). Therefore, the progression of the disease and HCV infectivity may be influenced by a diet including dairy products. Functional foods and their ingredients are known to be capable of modulating various biological processes such as apoptosis and have been attracting interest as natural resources for the prevention and treatment of cancer[10,11,40]. Dietary polyphenols derived from various fruits and vegetables have been suggested to be effective in cancer prevention. Although the importance of functional food ingredients as DAAs against HCV is not fully recognized, these findings suggest that they contribute to the elimination of the virus.

Several functional food ingredients have been reported to interfere with different steps of the HCV life cycle. Epigallocatechin-3-gallate (EGCG) (Figure 2A) and curcumin (Figure 2B), which are ingredients of green tea (Camellia sinensis) and the Indian spice turmeric (Curcuma longa), respectively, inhibit the entry of HCV into host cells[41,42]. Quercetin (Figure 2C), a flavonoid that is abundantly contained in onions, apples, berries, and red wine, has been shown to inhibit NS3 protease activity[43]. Punicalagin (Figure 2D) and its related substance punicalin from the pomegranate (Punica granatum L.) reduced the replication of HCV[44]. Naringenin (Figure 2E) from the grapefruit (Citrus X paradisi Macfady.) has been identified as an ingredient that interferes with viral assembly[45,46]. Diosgenin (Figure 2F) and epicatechin (Figure 2G), which are contained in yams (Dioscorea spp.) and

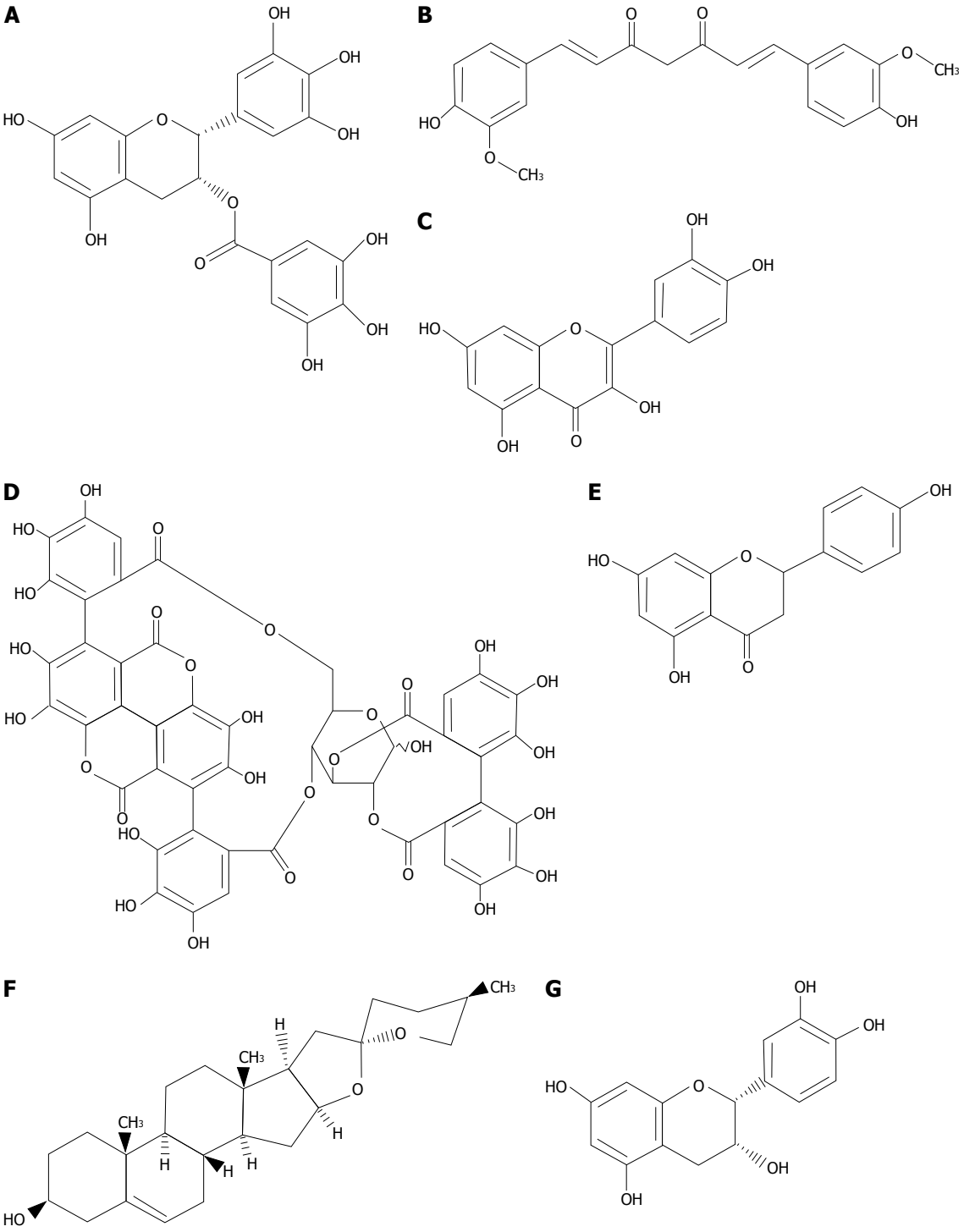

Figure 2 Chemical structure of functional food ingredients with anti-hepatitis C virus activities. A: Epigallocatechin-3-gallate; B: Curcumin; C: Quercetin; D: Punicalagin; E: Naringenin; F: Diosgenin; G: (-)-epicatechin.

green tea, respectively, also affect the signal transduction pathways of host cells and inhibit HCV replication *via* the signal transducer and activator of transcription 3 and cycloxygenase-2 pathways, respectively[47,48]. The finding that curcumin and quercetin also inhibited HCV replication by associating with sterol regulatory element binding protein-1 and heat shock proteins, respectively, indicated the existence of multifunctional ingredients[49,50]. Silymarin, which is an extract from milk thistle (Silybum marianum) and consists of at least 7 flavonoid compounds, was also found to interfere with several steps of HCV infectious process, such as NS5B polymerase activity and virus entry and transmission[51]. As shown in Figure 2, most ingredients are polyphenol compounds and, EGCG (A), quercetin (C), naringenin (E), and epicatechin (G) have similar chemical structures. There may be a characteristic structure modulating viral proteins and their associations with host proteins.

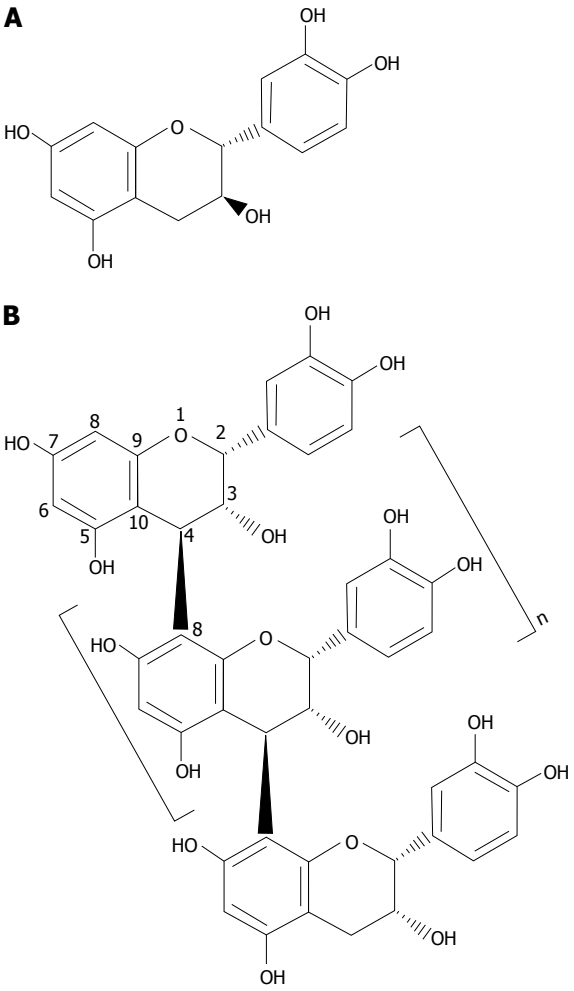

Figure 3 Chemical structures of a flavan-3-ol and proanthocyanidin. A: (+)-catechin; B: An example of a procyanidin B-type polymer with an (-)-epicatechin based structure.

Clinically, the supplementation of vitamin group has been reported to increase SVR rates in chronic hepatitis C patients who underwent the standard therapy with pegylated α-interferon and ribavirin[52-54]. Regarding significant side-effects of the standard therapy, a tomato-based functional food abundant in natural antioxidants alleviated the severity of anemia caused by ribavirin and improved the tolerance to the drug[55].

OLIGOMERIC PROANTHOCYANIDIN FROM BLUEBERRY LEAVES HAS SUPPRESSIVE ACTIVITY AGAINST HCV SUBGENOME REPLICATION *IN VITRO*

To identify functional food ingredients effective for hepatitis C, we comprehensively screened the extracts of commonly ingested agricultural products (1700 samples from 283 species) grown in Miyazaki prefecture, Japan using an HCV replicon cell system[13]. Samples having high antioxidative activities were first selected irrespective of edible part or non-edible part, and then the inhibitory activities against HCV subgenomic RNA replication were examined using the system. We found that extracts of blueberry leaves significantly suppressed the replication. Furthermore, by comparing the inhibitory activities using leaves from various kinds of blueberry species, it was found that the leaves of rabbit-eye blueberry (Vaccinium virgatum Aiton) had the highest activity[13]. Rabbit-eye blueberry is cultivated in a region with a warm climate, such as the southern areas of Japan, including Miyazaki prefecture. Its leaves have been also reported to be good sources of polyphenols and natural antioxidants[56].

We identified oligomeric proanthocyanidin as the blueberry leaf-derived inhibitor of HCV subgenomic RNA replication[13]. Proanthocyanidin is a polyphenol and has polymerized structures in which more than two flavan-3-ol units such as catechin (Figure 3A) and epicatechin (Figure 2G) are covalently linked. Figure 3B shows an example of the chemical structure of proanthocyanidin. Proanthocyanidin possesses two interflavan bonds, in which the A-type and B-type have two bond linkages (C4→C8 and O7→C2) and one linkage (C4→C8 or C4→C6), respectively[57], and both types co-exist in proanthocyanidin from the rabbit-eye blueberry plant[13]. While catechin, epicatechin, EGCG, and dimers such as procyanidin B2 did not exhibit inhibitory activity against HCV subgenomic expression in our experimental system, proanthocyanidin oligomer having polymerization degree of 8 to 9 markedly inhibited this expression[13]. This finding suggested that the HCV inhibitory activity of oligomeric proanthocyanidin in the replicon assay may require an oligomerized structure.

Proanthocyanidins are abundantly contained in various plants and foods[58] and contribute to organoleptic properties such as bitterness and astringency[59]. Proanthocyanidin-containing foods and nutritional supplements are known to have benefits in health promotion. United States Department of Agriculture Database reported proanthocyanidin contents of various foods, showing that apple peel, red kidney beans, pinto beans, cacao beans, cocoa, grape seeds, several nuts (almonds, hazelnuts, pecans, and pistachios), sorghum, and cinnamon are proanthocyanidin-rich[60]. Blueberry fruits are also relatively proanthocyanidin-rich; however, the fruits did not show significant HCV inhibitory activity compared to the leaves (unpublished data). In the fruits, proanthocyanidin contents of monomer, dimer, trimer, 4-6mer, 7-10mer, and polymer with degrees of polymerization greater than 10mer are 3.46, 5.71, 4.15, 19.57, 14.55, and 129.05 mg per 100 g edible portion, respectively[60]. As the inhibitory activity required the oligomeric structure of proanthocyanidin having a polymerization degree of 8 to 9 but not polymer and fresh blueberry leaf contained 3000-4000 mg proanthocyanidins per 100 g total extracts[13], leaves but not fruits from blueberry are likely suitable for the prevention of HCV-related diseases. With regard to the oral uptake, oligomeric proanthocyanidin seems to elute off by boiling for cooking as shown with pint beans[60]. Therefore, oligomeric proanthocyanidin from blueberry

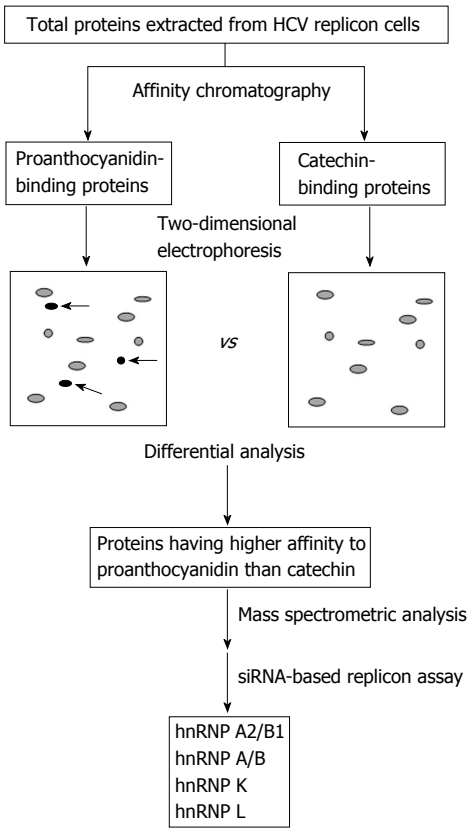

Figure 4 Identification strategy of candidate proteins involved in the proanthocyanidin-mediated inhibition of hepatitis C virus subgenomic expression[13]. Total proteins were extracted from hepatitis C virus (HCV) replicon cells and then proanthocyanidin-binding and catechin-binding proteins were purified by affinity chromatography using sepharose beads coupled with proanthocyanidin and catechin, respectively. Purified proteins were separated by two-dimensional electrophoresis followed by detecting spots of proteins having higher affinity to proanthocyanidin than catechin (arrows). Mass spectrometric analysis and further screening by a siRNA-based replicon assay showed that hnRNP A2/B1, A/B, K, and L are candidate proteins involved in the oligomeric proanthocyanidin-mediated inhibition of HCV subgenomic expression. hnRNP: Heterogeneous nuclear ribonucleoprotein.

leaves might be ingested as a hot water extract such as herbal tea. However, absorption efficiency of oligomeric proanthocyanidin in the intestine may be very low.

Proanthocyanidin has also been reported to possess anti-viral activity against other viruses, herpes simplex virus and human immunodeficiency virus type 1[61-65]. To the best of our knowledge, we first reported that the proanthocyanidin oligomer inhibited the expression of HCV subgenomic RNA[13]. However, the effects of oligomeric proanthocyanidin on HCV replication in hepatocytes *in vivo* currently remain unknown.

ACTION MECHANISM OF OLIGOMERIC PROANTHOCYANIDIN IN HCV REPLICON CELLS

The suppression of HCV subgenomic RNA replication by oligomeric proanthocyanidin has been attracting increasing attention. Polyphenolic compounds generally have high antioxidant activities[10,11,58]. Therefore, the nonspecific antioxidant activity of polyphenols may contribute to the suppression of HCV subgenomic RNA replication by oligomeric proanthocyanidin. However, we examined other polyphenolic compounds in our HCV replicon assay, and found that constitutional units such as catechin and epicatechin did not display suppressive activity, which requires the oligomerized structure of proanthocyanidin[13]. While it currently remains unknown whether proanthocyanidin oligomer can be translocated within the cells in spite of the structure, the ingredient has been reported to be absorbed from the digestive tract[66,67], implying the internalization into cells. Oligomeric proanthocyanidin appears to suppress HCV subgenomic RNA replication *via* a specific association with certain intracellular molecules.

Proteomic approach using two-dimensional differential gel electrophoresis combined with mass spectrometry provides a powerful tool to determine the cellular response to functional foods[40]. To clarify the action mechanism of oligomeric proanthocyanidin in HCV replicon cells, we performed proteomic analysis of proanthocyanidin-binding proteins purified by affinity chromatography[13]. Then, cellular proteins from replicon cells having higher affinity to proanthocyanidin than catechin were identified by a mass spectrometric analysis, and whether the proteins identified were associated with HCV RNA expression was further examined using a siRNA-based replicon assay (Figure 4). Four heterogeneous nuclear ribonucleoproteins (hnRNPs), hnRNP A/B, A2/B1, K, and L, were suggested to be possible cellular binding proteins of oligomeric proanthocyanidin. While siRNA targeting hnRNP A/B, K, and L showed weak inhibitory activities, the knockdown of hnRNP A2/B1 significantly suppressed HCV subgenomic replication[13].

HnRNPs comprise a family of RNA-binding proteins that are involved in diverse RNA-related biological processes[68]. They are multifunctional proteins composed of major and minor hnRNP proteins, and hnRNP A/B, A2/B1, K, and L that we identified belonged to the major hnRNPs[69]. Previous studies demonstrated that these hnRNPs regulated the metabolism of RNA such as pre-mRNA splicing and transcription[70-76]. For example, hnRNP A2/B1 was shown to affect the alternative splicing of several tumor suppressors and oncogenes in glioblastoma cells[72]. Furthermore, several studies reported interactions and cooperation between these hnRNPs[77-79]. hnRNP A2 and hnRNP L have also been shown to exist as a complex and regulate the expression of glucose transporter-1 by binding to mRNA 3'NTR[80,81].

In the HCV life cycle, hnRNPs are associated with HCV genome RNA and regulate its replication. hnRNP A1, which exhibits high homology with hnRNP A2/B1, was shown to facilitate HCV replication *via* binding to the HCV 5' and 3'NTRs (Figure 1), and the replication was significantly suppressed by the double knockdown of hnRNP A1 and hnRNP A2[82]. hnRNP K and hnRNP L are also NTR-binding proteins[83-85]. Furthermore, all

the hnRNPs we identified as the target protein candidates of oligomeric proanthocyanidin were included in HCV 3'NTR-binding proteins[86]. Collectively, these findings suggested that a complex composed of hnRNP A2/B1, A/B, K, and L may serve in HCV genome replication by binding to NTRs and oligomeric proanthocyanidin is an inhibitor of the replication complex. This possibility should be addressed in a further study.

CONCLUSION

Currently, a combination of pegylated recombinant interferons and ribavirin is used as the standard therapy for hepatitis C patients. Recently emerged DAAs are expected to provide new promising treatment options in hepatitis C patients. However, their high medical costs may make difficult to disseminate worldwide. We demonstrated that extracts of blueberry leaves suppressed HCV subgenome replication *in vitro*, and their active ingredient was oligomeric proanthocyanidin[13]. Investigations into the underlying action mechanism suggested that proanthocyanidin may be an inhibitor of several hnRNPs such as hnRNP A2/B1[13]. On the other hand, it currently remains unknown whether the oligomeric form of proanthocyanidin, which is required for the inhibition of HCV replication, can be efficiently absorbed from the digestive tract to maintain effective plasma concentrations *in vivo*. However, further basic research on the action mechanism of oligomeric proanthocyanidin against HCV replication may open ways to develop novel anti-HCV drugs and supplements for hepatitis C patients worldwide.

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TOPIC HIGHLIGHT

WJH 6th Anniversary Special Issues (7): Non-alcoholic fatty liver disease

Involvement of the TAGE-RAGE system in non-alcoholic steatohepatitis: Novel treatment strategies

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is a major cause of liver disease around the world. It includes a spectrum of conditions from simple steatosis to non-alcoholic steatohepatitis (NASH) and can lead to fibrosis, cirrhosis, liver failure, and/or hepatocellular carcinoma. NAFLD is also associated with other medical conditions such as obesity, diabetes mellitus (DM), metabolic syndrome, hypertension, insulin resistance, hyperlipidemia, and cardiovascular disease (CVD). In diabetes, chronic hyperglycemia contributes to the development of both macro- and microvascular conditions through a variety of metabolic pathways. Thus, it can cause a variety of metabolic and hemodynamic conditions, including upregulated advanced glycation end-products (AGEs) synthesis. In our previous study, the most abundant type of toxic AGEs (TAGE); *i.e.*, glyceraldehyde-derived AGEs, were found to make a significant contribution to the pathogenesis of DM-induced angiopathy. Furthermore, accumulating evidence suggests that the binding of TAGE with their receptor (RAGE) induces oxidative damage, promotes inflammation, and causes changes in intracellular signaling and the expression levels of certain genes in various cell populations including hepatocytes and hepatic stellate cells. All of these effects could facilitate the pathogenesis of hypertension, cancer, diabetic vascular complications, CVD, dementia, and NASH. Thus, inhibiting TAGE synthesis, preventing TAGE from binding to RAGE, and downregulating RAGE expression and/or the expression of associated effector molecules all have potential as therapeutic strategies against NASH. Here, we examine the contributions of RAGE and TAGE to various conditions and novel treatments that target them in order to prevent the development and/or progression of NASH.

Key words: Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Advanced glycation end-products; Toxic advanced glycation end-products; Receptor for advanced glycation end-products; Toxic advanced glycation end-products-receptor for advanced glycation end-products system; Diabetes mellitus; Cardiovascular disease; Dietary fructose; Dietary advanced glycation end-products

Core tip: Toxic advanced glycation end-products (TAGE) synthesis is increased by non-alcoholic steatohepatitis (NASH), and patients with NASH exhibit significantly increased serum and hepatic TAGE concentrations. Interactions between TAGE and the receptor for advanced glycation end-products (RAGE) have been suggested to cause oxidative stress and increase the fibrogenic potential of cultured human hepatic stellate cells. Therefore, TAGE signaling *via* RAGE and the resultant synthesis of reactive oxygen species might play a role in the worsening of hepatic pathology seen in NASH. These observations led us to suggest that extracellular and intracellular TAGE are involved in the pathogenesis of NASH.

Takeuchi M, Takino J, Sakasai-Sakai A, Takata T, Ueda T, Tsutsumi M, Hyogo H, Yamagishi S. Involvement of the TAGE-RAGE system in non-alcoholic steatohepatitis: Novel treatment strategies. *World J Hepatol* 2014; 6(12): 880-893 Available from: URL: http://www.wjgnet.com/1948-5182/full/v6/i12/880.htm DOI: http://dx.doi.org/10.4254/wjh.v6.i12.880

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a major cause of chronic liver disease in developed countries, and hence, is becoming a global public health issue[1]. NAFLD includes a range of conditions, from simple steatosis to non-alcoholic steatohepatitis (NASH)[2-4]. NASH has the potential to progress, which can result in cirrhosis, liver failure, and/or hepatocellular carcinoma[2-4]. NAFLD is regarded as a hepatic symptom of metabolic syndrome (MetS) and is associated with visceral obesity, abnormalities in glucose and lipid metabolism, insulin resistance (IR), and hypertension[5-7]. In NAFLD patients, underlying metabolic conditions such as those described above result in worsening liver dysfunction and a higher incidence of liver fibrosis and are also involved in the development of cardiovascular disease (CVD)[8,9].

Advanced glycation end-products (AGEs) might be involved in the mechanism that links NASH and diabetes mellitus (DM). Accumulating evidence indicates that in diabetic patients chronic hyperglycemia upregulates the production of AGEs (senescent macroprotein derivatives) *via* non-enzymatic glycation (the Maillard reaction). It has been demonstrated that the binding of AGEs to their receptor (RAGE) induces oxidative stress followed by inflammatory and/or thrombogenic responses in a variety of cell types. Furthermore, in diabetes such binding is considered to be involved in the pathogenesis and worsening of angiopathic conditions[10-16]. In our previous study, the most abundant type of toxic AGEs (TAGE); *i.e.*, glyceraldehyde-derived AGEs (Glycer-AGEs), were found to make a significant contribution to the development of angiopathic conditions in DM[17-20]. In addition, there is a growing consensus that TAGE-RAGE interactions affect gene expression, intracellular signaling, and the secretion of pro-inflammatory factors and induce reactive oxygen species (ROS) production in various cell types including hepatic stellate cells (HSC) and hepatocytes[21,22]. Thus, TAGE-RAGE interactions might play a role in the pathological changes associated with lifestyle-related diseases, particularly NASH. TAGE synthesis is increased in NASH, and NASH patients were found to exhibit significantly higher hepatic and serum TAGE concentrations than individuals with simple steatosis or healthy controls[23]. TAGE-RAGE interactions have also been found to be associated with the induction of oxidative stress and increases in the fibrogenic potential of cultured human HSC[22]. Therefore, it is suggested that TAGE signaling through RAGE and the subsequent ROS production play a role in the worsening of hepatic pathology observed in NASH.

Accordingly, inhibiting the binding of TAGE to RAGE and TAGE synthesis and downregulating RAGE expression and/or the expression of its effectors have potential as treatment strategies for NASH. Here, we examine the contributions of RAGE and TAGE to various conditions and novel treatments that target these molecules in order to prevent the development and/or progression of NASH.

AGEs

The Maillard reaction, in which the N-terminal α-amino or ε-amino regions of protein lysine residues react non-enzymatically with the ketone or aldehyde moieties of reducing sugars, *e.g.*, fructose, glucose, *etc.*, is responsible for synthesizing AGEs. AGEs are known to be involved in protein aging and the pathological complications associated with DM[10-13,17-20,24-27]. In hyperglycemic DM patients, the first step in this process involves the conversion of reversible Schiff base adducts to more stable covalently bound Amadori rearrangement products, which subsequently undergo further rearrangement to produce irreversibly bound moieties (AGEs), and this process can range in duration from days to weeks.

Initially, AGEs were identified based on their fluorescent yellow-brown appearance and their ability to produce cross-links with and between amino groups. However, the term AGEs now refers to numerous products associated with the advanced stages of the glycation process, including *N*-(carboxyethyl)lysine, *N*-(carboxymethyl)lysine (CML), and pyrraline, which are colorless and can not form cross-links with proteins[24-29]. *In vivo* AGE production is affected by the sugar concentration, the rate of turnover of the chemically modified target, and the time available. Increases in the glucose concentration were previously considered to have a major influence on the Maillard reaction; however, glucose is one of the least reactive sugars found in biological organisms[24,30]. As well as extracellular AGE synthesis, the rapid intracellular production of AGEs from intracellular precursors such as trioses, dicarbonyl compounds, and

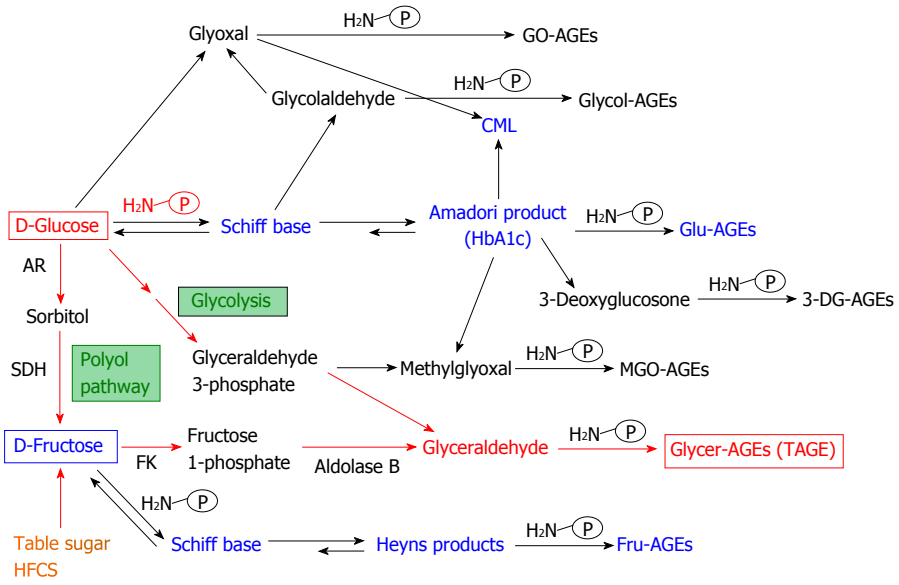

Figure 1 Alternative *in vivo* advanced glycation end-product synthesis routes. Reducing sugars, such as glucose, fructose and glyceraldehyde, are known to react non-enzymatically with the amino groups of proteins to form reversible Schiff bases and Amadori product/Heyns products. These early glycation products undergo further complex reactions such as rearrangement, dehydration, and condensation to become irreversibly cross-linked, heterogeneous fluorescent derivatives termed advanced glycation end-products (AGEs). Glu-AGEs: Glucose-derived AGEs; Fru-AGEs: Fructose-derived AGEs; Glycer-AGEs: Glyceraldehyde-derived AGEs; Glycol-AGEs: Glycolaldehyde-derived AGEs; MGO-AGEs: Methylglyoxal-derived AGEs; GO-AGEs: Glyoxal-derived AGEs; 3-DG-AGEs: 3-deoxyglucosone-derived AGEs; CML: N-(carboxymethyl)lysine; P-NH_2: A free amino residue; HbA1c: Hemoglobin A1c; TAGE: Toxic AGEs; HFCS: High-fructose corn syrup; AR: Aldose reductase; SDH: Sorbitol dehydrogenase; FK: Fructokinase.

fructose has been gaining attention[31,32]. Due to the great degree of variation in the structures of the AGEs found *in vivo* and the complex nature of the reactions required for their synthesis, only some AGEs have had their structures identified[33]. Furthermore, even the structures of cytotoxic AGEs are yet to be elucidated.

In a previous study, we found that α-hydroxyaldehydes (glycolaldehyde and glyceraldehyde), fructose, glucose, and dicarbonyl compounds (glyoxal and methylglyoxal, 3-deoxyglucosone) all contribute to protein glycation[27,34-37]. A total of 7 immunochemically distinct AGEs classes [methylglyoxal-derived AGEs; Glycer-AGEs; fructose-derived AGEs; glucose-derived AGEs (Glu-AGEs); 3-deoxyglucosone-derived AGEs; glyoxal-derived AGEs; and glycolaldehyde-derived AGEs] were found in serum samples collected from hemodialysis patients with type 2 DM (T2DM)[27,34-37]. Accordingly, we suggested that the *in vivo* formation of AGEs occurs *via* a process involving the Maillard reaction, sugar autoxidation, and sugar metabolism pathways (Figure 1).

PATHWAY FOR THE *IN VIVO* SYNTHESIS OF GLYCER-AGEs

In vivo, two different pathways are responsible for glyceraldehyde (GLA) production, (1) the fructose metabolic pathway (fructolysis) and (2) the glycolytic pathway (glycolysis)[18-20,38]. In pathway (1) under hyperglycemic conditions a rise in the intracellular glucose concentration stimulates the production of fructose *via* the polyol pathway in insulin-independent tissues, such as nerve tissue, the kidneys, the lens of the eye, red blood cells, and the brain[39-42]. In addition, fructose is a constituent of sucrose and high-fructose corn syrup (HFCS), and hence, is included in many people's diets[43,44]. Fructokinase phosphorylates fructose to fructose 1-phosphate, which is then broken down into dihydroxyacetone phosphate and GLA by aldolase B[45,46]. Next, the resultant GLA is transported (or leaks passively) across the cell membrane. GLA induces TAGE synthesis in the both intracellular and extracellular compartments; as for pathway (2) the enzyme glyceraldehyde 3-phosphate (G3P) dehydrogenase (GAPDH) usually breaks down the glycolytic intermediate G3P. However, reductions in GAPDH activity lead to the intracellular accumulation of G3P. As a result, G3P metabolism starts to occur *via* an alternative pathway, leading to a rise in the concentration of GLA, which promotes the synthesis of Glycer-AGEs, a major form of TAGE. This indicates that a positive feedback mechanism is in operation; namely, that the inhibition of GAPDH activity by TAGE promotes TAGE synthesis (Figure 2).

DIETARY FRUCTOSE

It is suspected that fructose is at least partially responsible for the obesity epidemic affecting developed countries. The greater prevalence of fructose in people's diets results in greater glucose flux and elevated fructose metabolism in hepatocytes. Fructose used to be considered to be a beneficial dietary substance due to the fact it does not stimulate insulin secretion; however, as insulin signaling plays a key role in the development of NAFLD, this property of fructose might be undesir-

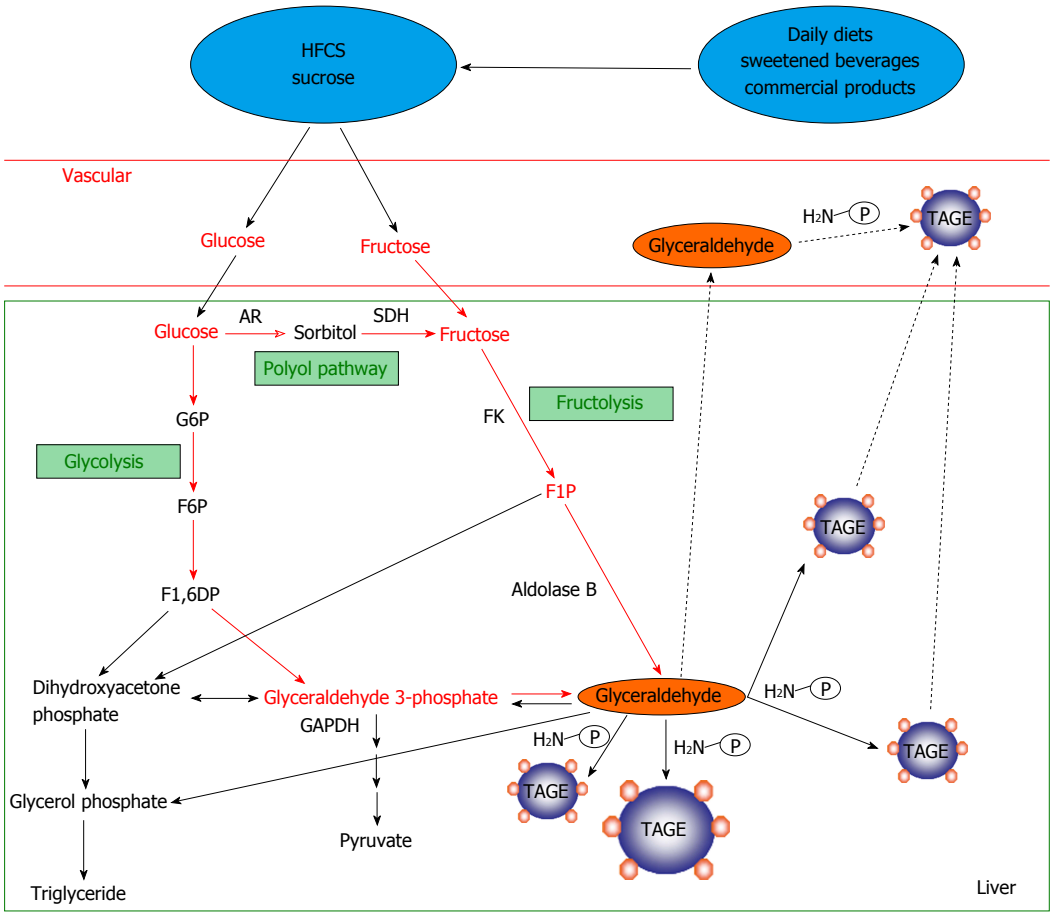

Figure 2 Routes for *in vivo* glyceraldehyde-derived advanced glycation end-products synthesis. The glycolytic intermediate glyceraldehyde 3-phosphate (G3P) is usually catabolized (glycolysis) by the enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH). However, reductions in GAPDH activity lead to the intracellular accumulation of G3P. As a result, G3P metabolism starts to occur *via* an alternative pathway, leading to a rise in the concentration of glyceraldehyde, which promotes the synthesis of TAGE. Fructokinase phosphorylates fructose to fructose 1-phosphate, which is then broken down into dihydroxyacetone phosphate and glyceraldehyde by aldolase B (fructolysis). The resultant glyceraldehyde is transported (or leaks passively) across the cell membrane. Glyceraldehyde promotes the formation of TAGE both intracellularly and extracellularly. AGEs: Advanced glycation end-products; TAGE: Toxic AGEs (glyceraldehyde-derived AGEs); HFCS: High-fructose corn syrup; AR: Aldose reductase; SDH: Sorbitol dehydrogenase; FK: Fructokinase; G6P: Glucose 6-phosphate; F6P: Fructose 6-phosphate; F1,6DP: Fructose 1,6-diphosphate; F1P: Fructose 1-phosphate; P-NH2: Free amino residue.

able[47-49]. In adolescents, increased fructose consumption is linked with various CVD risk factors. However, visceral obesity might be responsible for these associations. In the United States, fructose consumption is considered to be associated with the recent rise in the prevalence rates of obesity, fatty liver, and T2DM. The liver is extremely sensitive to variations in dietary content and plays the primary role in the metabolism of simple sugars, such as fructose and glucose[47,48].

The number of calories an individual consumes each day can have a significant influence on their risk of developing NAFLD because excessive energy intake results in obesity, leading to a greater risk of NAFLD. However, the development and progression of NAFLD are also affected by dietary composition. Of all carbohydrates, fructose plays an especially important role in NAFLD progression[50-53]. For example, it has been suggested that fructose consumption is associated with hepatic fat accumulation, fibrosis, and inflammation[54]. The accumulation of visceral adipose tissue and higher plasma triglyceride concentrations have also been linked with fructose consumption[55,56]. Thus, fructose has an important influence on the development of fatty liver disease[57].

Particular dietary sugars (especially fructose) are considered to play a role in the development and progression of NAFLD. The sugar additives (usually HFCS or sucrose) found in beverages and processed foods are widely viewed as the main source of the increased amounts of fructose consumed in developed countries. Dyslipidemia, obesity, and IR have all demonstrated strong associations with greater fructose consumption, and evidence indicating that fructose is involved in the development and progression of NAFLD is accumulating. Human studies have linked fructose consumption to hepatic fat accumulation, fibrosis, and inflammation. At present, it is unclear whether fructose can cause NAFLD on its own or whether it only promotes the condition when consumed in excessive amounts by individuals with a sedentary lifestyle, IR, and/or a positive energy balance. However, there is enough evidence to support a recommendation that the consumption of foods and drinks that are high in added fructose-containing sugars should

be limited[54,58].

Although we need to increase our knowledge regarding the influence of fructose on NAFLD, the links between excessive fructose consumption and hypertriglyceridemia, IR, and the accumulation of visceral adipose tissue are sufficiently clear to support a clinical recommendation that NAFLD patients decrease the amount of fructose in their diets.

AGE RECEPTORS

A variety of signaling pathways are activated by AGE synthesis *via* a series of cell surface receptors. Among AGE receptors, the multi-ligand receptor RAGE has been studied most extensively[59-63]. In addition, various other AGE receptors such as AGE-receptor complexes (AGE-R1/OST-48, AGE-R2/80K-H, and AGE-R3/galectin-3)[64,65] and certain members of the scavenger receptor family (SR-A[66], SR-B:CD36[67,68], SR-BI[69], SR-E: LOX-1[70], FEEL-1, and FEEL-2[71]) have been reported. It was reported that the expression of these AGE receptors varies between different types of cells or tissues and is influenced by metabolic changes, *e.g.*, changes associated with hyperlipidemia, DM, or aging[72]. *In vivo* and *in vitro* experiments examining the mechanisms responsible for the effects of AGEs and the factors that regulate their actions, *e.g.*, soluble RAGE (sRAGE), it was suggested that these molecules have significant pathobiological effects[63,73]. A variety of different cell types, such as neurons, hepatocytes, endothelial cells (EC), HSC, microglia, and pericytes, express RAGE[59-61].

In recent *in vitro* and *in vivo* studies, we found that protein amino moieties readily react with GLA to produce TAGE[18-20]. Furthermore, TAGE induce vascular inflammation and ROS production, and hence, promote the development of atherosclerosis in DM[74,75]. As TAGE display the greatest affinity for RAGE[74,75] and the binding of TAGE to RAGE adversely affects the vasculature of diabetic patients[18-20], TAGE might contribute to the greater CVD incidence rates seen in DM patients and impaired glucose tolerance (IGT) patients that display postprandial hyperglycemia. Furthermore, we have recently reported that in DM patients TAGE make significant contributions to the pathogenesis of angiopathy[19,20]. Accumulating evidence indicates that TAGE-RAGE interactions induce oxidative stress in various cell types, such as HSC and hepatocytes.

THE TAGE-RAGE SYSTEM IS INVOLVED IN LIVER DISEASE

As for the effects of TAGE on hepatocytes, we demonstrated that in Hep3B cells, a human hepatocellular carcinoma cell line, TAGE-RAGE interactions upregulated the hepatic production of C-reactive protein (CRP) by activating Rac-1[76]. The latter study indicated that at least two CRP expression-inducing signaling pathways are in operation in TAGE-treated Hep3B cells: the nuclear factor kappa B (NF-κB)-Rac-1-induced signal transducer and activator of transcription 3-dependent pathway, which is not directly affected by ROS, and an NADPH oxidase-mediated ROS-dependent pathway involving Rac-1[76]. During the induction of CRP expression by TAGE, the early stages of the process might be ROS-independent, whereas the latter stages might involve a ROS-mediated pathway. In Hep3B cells, the phosphorylation of insulin receptor substrate-1 (IRS-1) at its serine-307 residue and of c-Jun N-terminal kinase (JNK), c-JUN, and IκB kinase were promoted by TAGE. The increased phosphorylation of IκB kinase was associated with reductions in the concentration of IκB[77]. These effects of TAGE on Hep3B cells were abrogated by the overexpression of the dominant negative form of Rac-1. Treatment with curcumin, an inhibitor of NF-κB, or a JNK inhibitor decreased the phosphorylation of IRS-1 at its serine-307 residue in Hep3B cells. In addition, TAGE downregulated the tyrosine phosphorylation of IRS-1, weakened the affinity of the p85 subunit of phosphatidylinositol 3-kinase for IRS-1, and decreased glycogen synthesis in insulin-treated Hep3B cells. All of these effects were abrogated by treatment with NF-κB or JNK inhibitors[77]. Taken together, these results suggest that TAGE activate Rac-1, leading to the induction of the JNK- and IκB kinase-dependent serine phosphorylation of IRS-1, which in turn contributes to hepatic IR.

As the main producers of extracellular matrix molecules in the liver, HSC are important contributors to liver fibrogenesis[78]. In a previous study, we found that TAGE promoted the expression of genes and proteins associated with fibrogenesis or inflammation, *e.g.*, collagen type Iα2, monocyte chemoattractant protein-1 (MCP-1), and transforming growth factor-β1, in cultured HSC *via* NADPH oxidase-dependent ROS generation[22]. These results increase our knowledge of the role played by TAGE in the pathogenesis of NASH.

INTRACELLULAR TAGE ARE INVOLVED IN LIVER DAMAGE

GLA is a precursor of TAGE. Two GLA-forming pathways are considered to be in operation in the liver: (1) the glycolytic pathway and (2) the fructose metabolic pathway[18-20,38]. As a result, the liver tends to accumulate GLA to a greater extent than other organs.

Abnormalities in fructose and glucose metabolism can result in elevated intracellular GLA levels, which in turn can lead to upregulated intracellular TAGE synthesis, and such processes might play a role in the development of NASH. We found that in Hep3B cells GLA caused the intracellular TAGE concentration to rise and induced apoptosis in a concentration- and time-dependent manner[79]. Conversely, intracellular TAGE production was downregulated and GLA-induced apoptotic cell death was prevented by the addition of aminoguanidine, which inhibits AGE synthesis. Hepatocyte apoptosis was reported to be a characteristic of NASH in previous studies[80,81].

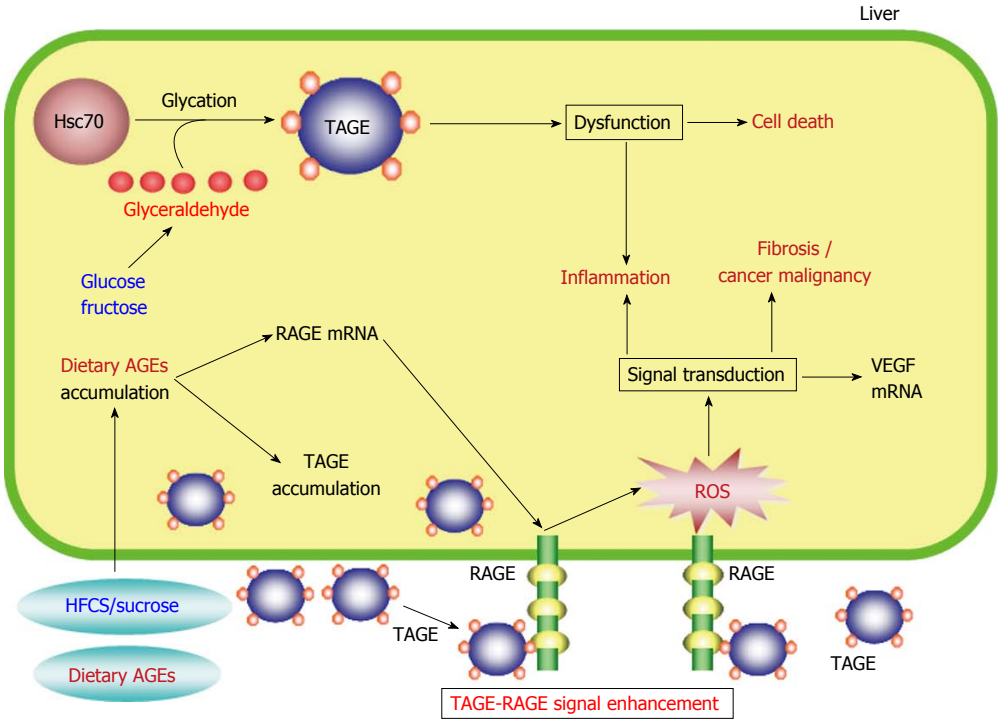

Figure 3 Proposed model for toxic advanced glycation end-products-mediated responses in the liver. HFCS/sucrose and dietary AGEs, which are normally found in sweetened beverages and commercial food products, are taken into the body, where they enhance the production/accumulation of TAGE, upregulate RAGE mRNA expression, and increase serum TAGE concentrations, leading to TAGE-RAGE interactions. The interaction between TAGE and RAGE alters intracellular signaling, gene expression, and the release of pro-inflammatory molecules and also induces oxidative stress in hepatocytes and hepatic stellate cells, which might contribute to the pathological changes observed in NAFLD/NASH. The formation of intracellular TAGE is associated with protein dysfunction followed by inflammation and cell death. Extracellular TAGE induce inflammation and fibrosis/cancer malignancy *via* RAGE signaling. AGEs: Advanced glycation end-products; TAGE: Toxic AGEs; RAGE: Receptor for AGEs; Hsc70: Heat shock cognate 70; ROS: Reactive oxygen species; VEGF: Vascular endothelial growth factor; HFCS: High-fructose corn syrup; NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis.

We identified a TAGE-modified protein (approximately 70 kDa) that was initially observed and tended to accumulate in GLA-treated hepatocytes as heat shock cognate 70 (Hsc70)[79]. Hsc70 might be important for GLA-induced cytotoxicity, as TAGE modifications have been demonstrated to have deleterious effects on protein function[82,83]. Furthermore, we found that the mRNA expression level of the acute phase reactant CRP was upregulated by intracellular TAGE[79]. Recently, it was demonstrated that NASH patients have higher plasma high-sensitivity CRP (hs-CRP) concentrations than healthy subjects or patients with simple steatosis[84,85]. Interestingly, in NASH patients a strong correlation was detected between the plasma hs-CRP concentration and the severity of liver damage[84,85]. In addition, intracellular TAGE were reported to induce inflammation, which is a characteristic of NASH. Taken together, these results indicate that intracellular TAGE make a significant contribution to the pathogenesis of NASH and might have potential as targets of treatments for NASH (Figure 3).

SERUM TAGE CONCENTRATIONS AND LIVER DISEASE

We measured the serum concentrations of three AGEs (Glu-AGEs, CML, and TAGE) in 66 patients with histologically defined-NASH who were free from liver cirrhosis, 10 patients with simple steatosis, and 30 control subjects to examine whether evaluating circulating AGE concentrations is useful for differentiating between NASH and simple steatosis[23]. The results of the latter study suggested that serum TAGE concentrations are involved in the pathogenesis of NASH and might be useful as biomarkers for differentiating between NASH and simple steatosis as: (1) The NASH patients exhibited significantly increased serum TAGE concentrations compared with the patients with simple steatosis and the healthy controls. According to receiver operating characteristic curves of the subjects' circulating TAGE concentrations, the optimal cut-off value for predicting NASH was 8.53 units/mL, which resulted in sensitivity and specificity values of 66.7% and 88.9%, respectively; (2) The subjects' homeostatic model assessment of insulin resistance (HOMA-IR) values and serum adiponectin concentrations (adiponectin is synthesized by adipose tissue and is an anti-inflammatory adipokine that can increase insulin sensitivity) exhibited positive and inverse correlations with their serum TAGE concentrations, respectively; (3) The subjects' serum TAGE concentrations were not correlated with the severity of their hepatic steatosis or fibrosis, nor were they influenced by the subjects' glucose tolerance status. The serum TAGE concentrations of the normal and IGT patients did not differ; (4) The NASH patients' hepatocytes contained TAGE,

whereas those belonging to the patients with simple steatosis exhibited negligible TAGE concentrations; and (5) The subjects' Glu-AGE and CML concentrations did not differ among the groups[23]. The above results indicate that serum TAGE concentrations are useful biomarkers for assessing residual liver function.

PUTATIVE MOLECULAR MECHANISMS RESPONSIBLE FOR THE ASSOCIATION BETWEEN NAFLD AND CARDIOVASCULAR DISEASE

Endothelial progenitor cells (EPC) help to maintain the structure and function of the endothelium, and hence, facilitate angiogenesis and vascular repair. In addition, the number of circulating EPC and their activity levels were found to be inversely correlated with atherosclerotic risk factors. Thus, the number and activity levels of EPC might be useful biomarkers for predicting cardiovascular events. In a recent study, Chiang *et al*[86] demonstrated that compared with the controls NAFLD patients had significantly fewer circulating EPC and the function of their EPC was impaired. Thus, in NAFLD patients reductions in the number of EPC or their activity might increase the likelihood of cardiovascular events.

In recent studies, we found that: (1) the serum concentration of TAGE, but not CML, was independently associated with HOMA-IR in non-diabetic subjects[87]; (2) in T2DM patients, the serum TAGE concentration, but not those of Glu-AGEs or hemoglobin A1c (HbA1c), can be used as a biomarker of cumulative postprandial hyperglycemia[88]; (3) the serum concentration of TAGE, but not those of HbA1c or CML, was demonstrated to be an independent predictor of vascular inflammation (as evaluated by [^{18}F] fluorodeoxyglucose-positron emission tomography in outpatients who visited Kurume University Hospital)[89]; (4) in healthy subjects, the serum TAGE concentration was found to be independently associated with a reduction in the number of circulating EPC and the impairment of the migratory activity of EPC[90]; and (5) a Japanese trial assessing the utility of pitavastatin and atorvastatin as treatments for acute coronary syndrome reported that high baseline TAGE concentrations were associated with plaque progression[91]. These results suggested that the serum TAGE concentration, but not those of HbA1c, CML, or Glu-AGEs, might be a useful biomarker for predicting atherosclerosis progression and future cardiovascular events. Thus, TAGE-RAGE system activation is considered to lead to a greater risk of cardiovascular events and contribute to the progression of liver damage, which would provide a mechanism link between CVD and NAFLD/NASH.

NOVEL TREATMENT STRATEGIES

The majority of studies about NASH have attempted to assess the relationships between NAFLD/NASH and T2DM, CVD, or chronic kidney disease (CKD)[92]. The results outlined above strongly suggest that the TAGE-RAGE system is involved in the development and progression of NASH. As a result, several therapeutic strategies that target this system, *e.g.*, inhibiting TAGE synthesis, downregulating the expression of RAGE or molecules involved in its downstream pathways, and blocking TAGE-RAGE interactions, have been developed as potential treatments for NASH.

The inhibition of TAGE synthesis: Acarbose

Whilst there are many drugs that are able to improve glycemic control, including patients' postprandial plasma glucose concentrations, some drugs specifically target postprandial hyperglycemia.

The absorption of carbohydrates from the small intestine can be delayed by treatment with the α-glucosidase inhibitor acarbose, and T2DM patients that were administered acarbose displayed less severe postprandial hyperglycemia[93]. A recent study found that in patients with T2DM or IGT acarbose treatment reduced the rate at which the intimal media of the carotid arteries thickened and led to a lower incidence of CVD[93], indicating that acarbose ameliorates postprandial hyperglycemia, and hence, inhibits the development and progression of CVD. In an *in vivo* study, we found that protein amino moieties readily react with GLA to produce TAGE, leading to oxidative stress and vascular inflammation. These observations suggested that in DM GLA plays a role in promoting the development of atherosclerosis[18-20]. In a study involving T2DM rats, we demonstrated that the serum concentration of TAGE, but not HbA1c, is a marker of cumulative postprandial hyperglycemia[94]. Based on the abovementioned results, we suggest that acarbose reduces serum TAGE concentrations, which could at least partially explain its cardioprotective effects *in vivo*. In a previous study, 50 mg acarbose (dosing schedule: thrice a day for a 12-wk period) were administered to 13 Japanese T2DM patients who were free from inflammatory conditions, atherosclerotic heart disease, and microangiopathy and had never taken oral hypoglycemic agents. The patients' serum TAGE concentrations as well as their serum levels of other biological molecules were assessed before and after the administration of acarbose[88]. The DM patients' serum free fatty acid and TAGE concentrations had fallen significantly after 12 wk' acarbose treatment. Acarbose also reduced their postprandial plasma glucose concentrations. These results indicate that HbA1c concentrations might not accurately reflect the ameliorative effects of acarbose on postprandial hyperglycemia. Furthermore, they suggest that serum TAGE concentrations might be useful biomarkers for assessing cumulative postprandial hyperglycemia in T2DM patients. As TAGE have adverse effects on CVD[20], acarbose might be useful for preventing CVD in NASH patients with T2DM or postprandial hyperglycemia.

Inhibiting the binding of TAGE to RAGE using sRAGE

RAGE was found to contribute to acute liver damage in

numerous studies, and the blockade of RAGE was demonstrated to reduce cholestatic, toxic, and ischemic liver damage[95-98].

Patients with chronic liver injuries were found to exhibit significantly higher hepatic RAGE expression levels[99], and in NAFLD patients a correlation was detected between the severity of fibrosis and the patients' serum TAGE concentrations, indicating that RAGE and TAGE make significant contributions to the development of liver disease[23]. In addition, DM, which upregulates AGE synthesis and RAGE expression, has been found to accelerate the progression of fibrosis in a number of human liver conditions, including chronic hepatitis C and NAFLD[100]. Recently, we found that TAGE-RAGE interactions promote inflammation, affect the expression levels of various genes and the activity of intracellular signaling pathways, and induce oxidative stress in various kinds of cells. These effects might be involved in the pathological changes seen in various chronic diseases[17-20].

Endogenous sRAGE has recently been detected in humans[74]. It has been suggested that it is synthesized *via* the cleavage of a splice variant of RAGE (a type of secretory RAGE exhibiting C-terminal truncation) or full-length cell surface RAGE[74]. Patients with T1DM or T2DM display increased total endogenous sRAGE concentrations[101-104]. In addition, we and others have detected positive correlations between the total serum sRAGE concentration and serum TAGE concentrations in both non-DM and DM subjects[104,105]. Furthermore, body mass index-, sex-, and age-adjusted TAGE concentrations were found to increase significantly in proportion to the rise in the serum sRAGE concentration in non-DM subjects[104,105]. These results indicate that *in vivo* circulating sRAGE, which functions as a decoy receptor, is unable to bind to and remove the TAGE present in the blood in an efficient manner. As TAGE promote RAGE expression, the blood sRAGE concentration might be a marker of RAGE production within tissues. Furthermore, it might change in response to variations in the serum concentration of TAGE in order to ameliorate TAGE-induced tissue damage including NASH[106-109].

An angiotensin Ⅱ type 1 receptor blocker: Telmisartan

It has been suggested that the TAGE-RAGE axis interacts with the renin-angiotensin system. In a previous study, we suggested that the angiotensin Ⅱ type 1 receptor blocker telmisartan reduces RAGE expression *via* its ability to modulate the peroxisome proliferator-activated receptor-γ (PPAR-γ)[21,110]. We came to this conclusion due to the following observations, which were obtained in experiments involving Hep3B cells: (1) whilst telmisartan downregulated ROS synthesis, TAGE-induced RAGE expression, and CRP expression, candesartan did not induce any of these processes; (2) the PPAR-γ inhibitor GW9662 abrogated the telmisartan-induced inhibition of the expression of RAGE and its associated effector molecules; (3) the effects of ciglitazone and troglitazone, which are full agonists of PPAR-γ, were similar to those of telmisartan; and (4) the administration of curcumin, an inhibitor of NF-κB, or antioxidants abrogated the upregulation of CRP mRNA expression induced by TAGE. Due to its unique ability to modulate PPAR-γ, telmisartan is increasingly considered to be a useful cardiometabolic sartan[21,110,111]. In addition, it has been demonstrated that thiazolidinediones downregulate endothelial RAGE expression *via* NF-κB suppression[112]. These results suggest that telmisartan has anti-inflammatory effects on TAGE signaling; *i.e.*, it reduces hepatic RAGE expression by activating PPAR-γ, and might also help to protect against NASH.

A hydroxymethyl-glutaryl-CoA reductase inhibitor: Atorvastatin

In a recent study, we found that in Hep3B cells the hydroxymethyl-glutaryl-CoA reductase inhibitor atorvastatin reduced TAGE-induced ROS synthesis in a dose-dependent manner[113]. In addition, atorvastatin and the antioxidant N-acetylcysteine downregulated CRP expression at both the mRNA and protein levels in TAGE-treated Hep3B cells[113]. These results showed that the antioxidative effects of atorvastatin abrogate CRP expression-associated TAGE signaling. Furthermore, they indicate that statins protect blood vessels from damage and abrogate the adverse effects of TAGE by downregulating the activity of their effector molecules.

The consumption of fructose-containing beverages is associated with a greater risk of MetS-related conditions, including NAFLD. Despite the fact that caloric restriction and weight loss is the only effective treatment for NAFLD, it has been demonstrated that atorvastatin is safe for use in NAFLD patients and results in improvements in their hepatic histology. In a previous study, we found that atorvastatin reduced the serum TAGE concentrations of 43 patients with a combination of biopsy-proven NASH and dyslipidemia[114]. After 12 mo atorvastatin treatment (10 mg daily), all of the patients demonstrated significant reductions in their hepatic transaminase (aspartate aminotransferase and alanine aminotransferase (ALT) and γ-glutamyl transpeptidase concentrations. In addition, by end of the treatment their plasma tumor necrosis factor-α (TNF-α) and plasma adiponectin concentrations were reduced by 31% and elevated by 16%, respectively. The patients' HOMA-IR values were slightly reduced. The patients' liver/spleen ratios rose significantly from 0.54 ± 0.26 at the baseline to 0.94 ± 0.24 at the end of the treatment; however, their visceral fat area values were unchanged. During the treatment, the patients' serum TAGE concentrations fell significantly (they were 10.4 ± 3.8, 5.9 ± 3.3, and 2.5 ± 1.1 units/mL before the treatment and after 6 mo and 12 mo treatment, respectively). Correlations were detected between the patients' serum TAGE concentrations and their serum concentrations of thiobarbituric acid reactive substances (TBARS), TNF-α, procollagen type Ⅲ propeptide, ALT, and type Ⅳ collagen 7S[114].

The administration of atorvastatin to Sprague-Dawley

male rats that had consumed a liquid fructose solution (10% w/v) abrogated the inflammatory and metabolic changes induced in the liver by fructose. These beneficial effects were considered to be due to the anti-inflammatory activity of atorvastatin and its downregulation of the hepatic expression of fructokinase, which inhibits fructose metabolism in the liver[115]. Reduced synthesis of GLA (a TAGE precursor and a fructose metabolite) leads to a drop in TAGE synthesis. Atorvastatin is able to reduce the serum TAGE concentration without altering glucose metabolism and does so in a cholesterol-lowering-independent manner. In the abovementioned study, the serum TAGE concentrations of the NASH patients with dyslipidemia fell significantly after the atorvastatin treatment, but their glucose metabolism was unaffected[114]. In conclusion, atorvastatin was demonstrated to be an effective treatment for NASH patients with dyslipidemia who did not respond adequately to diet and exercise therapy. In addition to improving their serum TAGE concentrations, atorvastatin also improved their histological and biochemical data. As atorvastatin decreased the serum TAGE concentrations of NASH patients with dyslipidemia, TAGE might be useful biomarkers for the treatment of NASH[114]. Controlled trials should be performed to further examine the clinical utility of TAGE as biomarkers in NASH.

Dietary AGEs: Kremezin

A study involving mice produced found that AGEs facilitate the progression from simple steatosis to NASH and liver fibrosis[116]. In the methionine choline-deficient rat model of NAFLD, high dietary consumption of AGEs results in elevated hepatic AGE concentrations and increased fibrosis, liver damage, and inflammation. The latter effects are considered to be mediated *via* the RAGE- and oxidative stress-dependent profibrotic effects of AGEs on activated HSC[117]. The above observations indicate that pharmacological and dietary strategies that target the AGE-RAGE system are able to slow the progression of NAFLD.

In a recent study, we detected increased hepatic expression levels of vascular endothelial growth factor (VEGF) and RAGE in rats that had been administered Glu-AGE-rich beverages. This suggested that dietary AGE consumption is associated with the hepatic expression of liver fibrosis-related genes[118]. Moreover, the abovementioned rats' livers were found to contain TAGE- and Glu-AGE-positive cells[118]. These results indicate that the consumption of Glu-AGE-rich beverages leads to upregulated hepatic expression of RAGE and VEGF and encourages the build-up of TAGE and Glu-AGEs, resulting in the binding of TAGE to RAGE. Thus, it is important to consider the amounts of Glu-AGEs present in foods to prevent liver disease, especially in people that are at risk of CKD, CVD, NAFLD/NASH, or DM.

It has been demonstrated that Kremezin, an oral adsorbent that consists of porous spherical carbonic particles, is able to attenuate the progression of chronic renal failure (CRF) by removing uremic toxins, *e.g.*, indoxyl sulfate precursors, from the intestine[119]. In CRF patients without DM, 3 mo Kremezin treatment (6 g/d) resulted in markedly reduced serum TAGE and Glu-AGEs concentrations, while the concentrations of these molecules were unaffected in renal function- and age-matched CRF patients that did not receive the drug[120]. The EC in the post-treatment serum samples collected from the Kremezin-treated patients exhibited markedly lower concentrations of MCP-1, vascular cell adhesion molecule-1, and RAGE mRNA than those found in the serum samples collected before treatment[120]. These findings indicate that the pathogenesis of vascular damage is influenced by dietary Glu-AGEs in TAGE-RAGE-related conditions and that reducing the amount of dietary Glu-AGEs taken into the body might represent a useful strategy against NAFLD/NASH.

Further clinical studies might provide insights into whether restricting the consumption of Glu-AGEs would be beneficial for preventing or slowing the progression of NAFLD/NASH and whether Glu-AGEs represent a novel therapeutic target for treatments that aim to reduce the risk of liver disease.

CONCLUSION

TAGE formation and accumulation are known to increase in various tissues during normal aging and to occur at a markedly accelerated rate in DM patients[18-20]. An increasing body of evidence suggests that TAGE are involved in the pathogeneses of various disorders including hypertension, Alzheimer's disease, diabetic vascular complications, CVD, NAFLD/NASH, and cancer growth and metastasis[7,8,18-23,79,87-91,114,121-126]. We found evidence that TAGE are involved in the pathogenesis of NASH in humans[7,23,114]. TAGE stimulated the proliferation and activation of HSC *in vitro via* RAGE, which resulted in hepatic inflammation and fibrosis[22]. In addition, NASH patients exhibited significantly higher serum TAGE concentrations than patients with simple steatosis or healthy controls[7,23]. Atorvastatin reduced the serum TAGE concentrations of NASH patients with dyslipidemia, and correlations were detected between the patients' serum TAGE concentrations and their serum TNF-α, ALT, type Ⅳ collagen 7S, procollagen type Ⅲ propeptide, and TBARS concentrations[114]. In a recent study, we found that non-B or non-C hepatocellular carcinoma (NBNC-HCC) patients had significantly increased circulating TAGE concentrations compared with NASH subjects without HCC and the control subjects[127]. The findings outlined in the present review indicate that TAGE contribute to the pathogenesis of NBNC-HCC and that they might be useful biomarkers for discriminating between NBNC-HCC and NASH.

In conclusion, an increasing amount of evidence indicates that TAGE and RAGE both make important contributions to liver disease. TAGE might play a role in the development and progression of NASH and could be

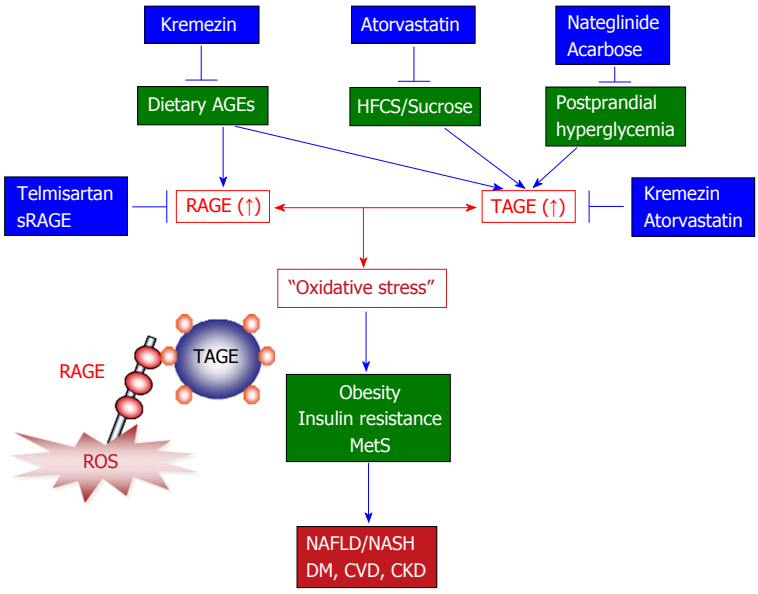

Figure 4 The toxic advanced glycation end-products-receptor for advanced glycation end-products system and novel treatments that target this system to prevent the development and/or progression of non-alcoholic steatohepatitis. Accumulating evidence suggests that TAGE-RAGE interactions affect intracellular signaling, gene expression, and the release of pro-inflammatory molecules and also induce oxidative stress in numerous types of cells, all of which have the potential to contribute to the pathological changes associated with lifestyle-related diseases including NAFLD/NASH. Since TAGE display the strongest binding affinities for RAGE and have adverse effects on diabetic vessels through their interactions with RAGE, TAGE might be partly responsible for the increased risk of cardiovascular disease (CVD) seen in diabetes mellitus (DM) patients and the impaired glucose tolerance observed in patients with postprandial hyperglycemia. NAFLD is considered to be a hepatic symptom of metabolic syndrome (MetS) and is strongly associated with insulin resistance, obesity, and abnormalities in glucose and lipid metabolism. It is important to consider the amounts HFCS/sucrose and AGEs present in foods to prevent liver disease, particularly in individuals that are at high risk of developing NAFLD/NASH, DM, CVD, or chronic kidney disease (CKD). Taken together, the present study suggests that TAGE could be used as novel therapeutic targets for the prevention of lifestyle-related diseases. Therefore, inhibiting the formation of TAGE, blocking TAGE-RAGE interactions, and suppressing the expression of RAGE or its downstream effectors all have potential as therapeutic strategies against lifestyle-related disease including NAFLD/NASH. AGEs: Advanced glycation end-products; TAGE: Toxic AGEs; RAGE: Receptor for AGEs; sRAGE: Soluble form of RAGE; NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; HFCS: High-fructose corn syrup.

useful biomarkers for differentiating between NASH and NAFLD or between NBNC-HCC and NASH. Further clinical and experimental studies are required to elucidate the mechanisms by which the TAGE-RAGE system affects the development and progression of lifestyle-related conditions including NAFLD/NASH (Figure 4).

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TOPIC HIGHLIGHT

WJH 6th Anniversary Special Issues (7): Non-alcoholic fatty liver disease

Transitions of histopathologic criteria for diagnosis of nonalcoholic fatty liver disease during the last three decades

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is the hepatic manifestation of metabolic syndrome, and is the most common type of chronic liver diseases in the majority of developed countries. NAFLD shows a wide spectrum of disorders including simple steatosis, nonalcoholic steatohepatitis (NASH), and cirrhosis. While simple steatosis is recognized to be benign and stable, NASH is considered to be an aggressive form of the disease progressing to cirrhosis. Currently, differentiation between NASH and simple steatosis can be done only by liver biopsy. Despite many proposals and revisions, the histological criteria for the differentiation have not been perfected yet. In this review article, the changes in the histopathologic criteria of NAFLD during the last three decades are summarized, and perspectives of the future changes are demonstrated. The discussion focuses on how pathologists have been dealing with "hepatocellular ballooning". Loose criteria, in which hepatocellular ballooning was not required for the diagnosis of NASH, were applied in many clinical studies published in around 2000's, whereas a strict criterion based on the presence/absence of hepatocellular ballooning was approved recently. Hence, simple and reliable methods of identifying ballooned hepatocytes are being sought. Clinical and pathological predictors of NAFLD-related hepatocarcinogenesis will also be sought in the future.

Key words: Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis; Hepatocellular ballooning; Cirrhosis; Hepatocellular carcinoma

Core tip: The differentiation between nonalcoholic steatohepatitis and simple steatosis can be done only by liver biopsy. Through many proposals and revisions, the histological criteria for the differentiation have been changed. The changes in the criteria during the last three decades are exhibited in this review article, with a special interest in "hepatocellular ballooning".

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INTRODUCTION

In newly proposed disease entities, or even in already established ones, the definitions and diagnostic criteria may be revised repeatedly. The revisions are led by alterations in recognition of the disease, changes in morbidity and social healthcare strategy in each era, elucidation of the pathologic mechanisms, *etc.* Hence, these changes probably occur more frequently in a disease of unknown etiology. Nonalcoholic fatty liver disease (NAFLD) and its aggressive form, nonalcoholic steatohepatitis (NASH),

Table 1 Histological criteria for diagnosis of nonalcoholic steatohepatitis used in the previous studies (1980-preset)

Ref.	Year	Steatosis	Inflammatory cell infiltration	Hepatocellular necrosis	Hepatocellular ballooning	Mallory-Denk body	Pericellular fibrosis
Ludwig *et al*[1]	1980	≥ Moderate	+	+			
Falchuk *et al*[23]	1980	≥ Moderate				+	+
Diehl *et al*[26]	1988	≥ Mild		+		+[1]	+[1]
Nagore *et al*[25]	1988	≥ Mild	+		+	+	+
Lee *et al*[35]	1989	≥ Mild	+				+
Powell *et al*[28]	1990	≥ Moderate	+				
Wanless *et al*[29]	1990	≥ 5%			+		
Bacon *et al*[36]	1994	≥ Minimal	+				
Laurin *et al*[37]	1996	≥ Minimal	+				
George *et al*[38]	1998	≥ Minimal	+				
Younossi *et al*[32]	1998	> 1/3	+	+			
Matteoni *et al*[33]	1999	> 1/3	+	+	+[2]		
Brunt *et al*[41]	1999	> 0%	+				
Dixon *et al*[44]	2001	≥ Mild	+	+	+[1]		+[1]
Neuschwander-Tetri *et al*[2]	2003	≥ 5%	+		+		
Bedossa *et al*[51]	2012	> 5%	+		+[3]		

[1]Either one; [2]Modified in 2009[34]; [3]Normal-sized hepatocyte with clear reticular cytoplasm. +: Required; Blank: Not required.

are representative examples.

Although NASH/NAFLD have generally been accepted as independent diseases since Ludwig's monumental publication in 1980[1], minor revisions regarding definition, criteria (mainly histopathologic features and a cutoff level of alcohol consumption) and diagnostic algorithm have continued to be made. A goal of the revisions is establishment of accurate selection criteria to extract NAFLD cases that are most likely to progress to cirrhosis or to hepatocellular carcinoma (HCC). The selected patients become subjects of follow-up and therapeutic interventions[2,3]. NAFLD is considered to be the most common chronic liver disease in the majority of developed countries, and clarification of the high-risk group of NAFLD patients is the most critical issue in current hepatology.

Noninvasive clinical methods, which can evaluate the degree of steatosis and can diagnose NAFLD in some cases, have been developed[4,5]. However, since they cannot evaluate inflammatory activity, the diagnosis of NASH still requires histological examination[4]. It is not possible to perform liver biopsy in every NAFLD patient, and thus, the detailed pathobiological and clinicopathologic characteristics of NASH/NAFLD have not yet been elucidated. Consequently, the histopathologic criteria for the diagnosis of NASH/NAFLD have changed repeatedly. The ambiguous and wandering criteria have confused general pathologists.

What are the reliable histopathologic markers of true NASH? No one can provide an appropriate answer to this substantial query. I review the 30-year history of the revision process that contained many trials and errors (Table 1). This review may not only introduce a clue to the answer, but also provide a direction for future studies on NASH/NAFLD.

BEFORE "LUDWIG" (-1979)

The proposal of NASH as a new disease entity by Ludwig *et al*[1] in 1980 was truly the first milestone in NASH/NAFLD research. Historically, many pathologists prior to Ludwig focused on fatty livers and cirrhosis associated with morbid obesity or diabetes[6-13]. Histologic pictures shown in the earlier reports were of NASH/NAFLD according to the current diagnostic criteria. They had noticed even some morphological features of this type of fatty livers rather different from alcoholic fatty livers, such as low percentages of Mallory-Denk body and siderosis and frequent nuclear glycogen[11-13]. However, due to the facts that most fatty livers did not progress to fibrosis and cirrhosis[13,14], and that livers could physiologically store a certain amount of lipid, there had been for a long time controversy regarding the pathologic significance of lipid accumulation in livers. In other words, fatty change was considered as an innocent bystander, not harmful, and an accompanying phenomenon caused by hepatotoxic pathogens[15].

There was no obvious definition of the physiological level of hepatic fat. Galambos *et al*[16] studied hepatic histopathologic findings corresponding to abnormal laboratory test results in obese patients. In that study, the authors defined > 33% fatty change as an abnormal/pathologic condition. There was no explanation about how the authors determined 33% as the normal limit. This fact indicates that the value of 33% was acceptable without any explanations as the normal limit at that time.

Undiscovered hepatitis C virus (HCV)[17] might have disturbed to recognize NASH/NAFLD as independent hepatic disorders. Especially HCV genotype 3 is now known to be able to cause prominent hepatic steatosis as well as necroinflammation[18]. Pathologists might have misunderstood NASH as viral hepatitis, and simultaneously, HCV-related hepatitis as primary steatotic liver disease. The potential overlap of NASH/NAFLD and HCV-related hepatitis is still a focus of debate[19,20].

In that era, earlier than Ludwig's, many reports concerning NASH/NAFLD were published from Japanese institutes[7,21,22]. Although the medical interest had not

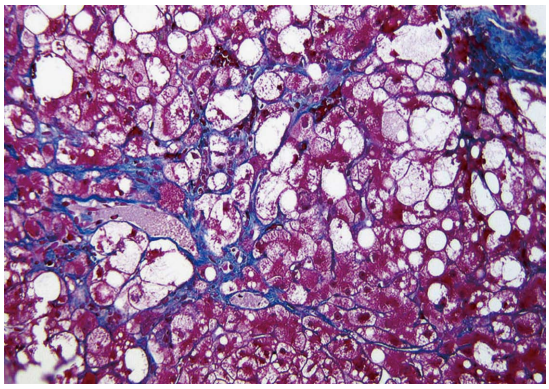

Figure 1 Typical case of nonalcoholic steatohepatitis showing hepatocellular ballooning and perivenular/pericellular fibrosis (Azan-Mallory stain; Original magnification, × 200).

been directed to metabolic syndrome, Japanese pioneer researchers investigated fatty liver disorders with keen observations and deep insights. Surprisingly, they suggested that fatty change was a first step of NAFLD progression to cirrhosis and dysfunctions of hepatocellular organelles including endoplasmic reticulum were pivotal[7,22]. These are completely identical with the present recognitions of pathological mechanisms of NASH/NAFLD.

AFTER "LUDWIG" (1980-1999)

The current disease concept and terminology of NASH were established only by a single pathologic report entitled "Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease" written by Ludwig *et al*[1] in 1980. At present, it is well recognized that the contribution of this breakthrough article to hepatology is too large to be estimated. The report consisted of clinicopathologic reviews of twenty cases of NASH. Their inclusion criteria, namely diagnostic criteria of NASH, were extremely simple and clear: non-habitual drinkers with liver damage that was indistinguishable from alcoholic injury histologically. The authors proposed to categorize all types of liver damages fulfilling the criteria into one disease entity named NASH. A little confusion might have arisen because NASH included fatty liver disorders associated with nutritional disturbances and even drug-induced damage as well as those associated with morbid obesity and diabetes. In addition, the definition of "nonalcoholic" became a big issue; they excluded only obvious alcohol abusers.

In the same year (1980), Falchuk *et al*[23] published their article entitled "Pericentral Hepatic Fibrosis and Intracellular Hyalin in Diabetes Mellitus", and suggested that an inflammatory hepatic disorder associated with diabetes mellitus was an intermediate illness between fatty liver and cirrhosis. The contents of the papers by Ludwig *et al*[1] and Falchuk *et al*[23] complemented each other, and emphasized the independence and importance of NASH among chronic liver diseases. However, the etiopathology of NASH was not elucidated, and the concept of NASH did not gain complete acceptance for about 20 years. As proof, some articles very similar to the earlier studies than 1980 were published, and different terms such as diabetic hepatitis and fatty liver hepatitis were used instead of NASH[24,25]. A study seeming to have repeated the Ludwig's original work also appeared. It was not surprising that the results of those studies validated the presence of the new disease, NASH, and its progressive nature[25,26].

A growing interest produced one substantial question: what kind of fatty liver disorders is truly progressive? Two scientific streams, which still influence today's research trends, sprang from the query. The diagnostic criteria in the streams have been gradually modified.

One of the scientific streams was to highlight specific findings of NASH/NAFLD. Clain *et al*[27] systematically reviewed previous papers on NAFLD, and concluded that the presence of perivenular/pericellular fibrosis (Figure 1) potentially indicated a progressive disease[9]. Powell *et al*[28] confirmed that NASH was actually a slowly progressing disease, and tried to classify NASH on the basis of steatosis, inflammation, Mallory-Denk bodies and fibrosis. However, they could not find a relationship with prognosis. Wanless *et al*[29] accentuated the importance of hepatocellular ballooning (Figure 1), and made a diagnosis of NASH according to the presence of steatosis and ballooning. In that article, histologically abnormal lipid accumulation was defined as fatty change that affected more than 5% of hepatocytes. There was no obvious evidence for the definition of "more than 5%". A previous biochemical analysis revealed that normal livers (livers of healthy persons) could store lipids comprising less than 5% of liver weight[30]. Accordingly, "more than 5%" has been used as a standard value for defining pathologic hepatic lipid accumulation until now. Teli *et al*[31] defined NASH as hepatic steatosis with lobular inflammation, hepatocellular ballooning, Mallory-Denk bodies, and hepatocellular necrosis. They suggested that non-NASH NAFLD (namely, simple steatosis, and steatosis with portal inflammation) did not progress to NASH and cirrhosis. This stream led to the NAFLD classification of Younossi *et al*[32] and to Matteoni's classification[33]. Hepatocellular ballooning was a key finding for their classification. Later they insisted on the necessity of hepatocellular ballooning in diagnosis of NASH (Table 1)[34]. In their original classification, however, they used a term "steatohepatitis" for type 2 NAFLD, which was steatosis with lobular inflammation but without ballooning.

The other scientific stream was to establish a score system based on semi-quantitative analyses of the histological severity of liver damage. The trial was initiated by Diehl *et al*[26], followed by Lee *et al*[35] They evaluated the degree of steatosis, inflammatory cell infiltration, hepatocellular damage, Mallory-Denk bodies, and fibrosis using four- or five-step scales. Unfortunately, they failed to find a relationship between the scores and prognoses. Bacon *et al*[36] also performed a semi-quantitative analysis using a system similar to Diehl's system, but did not described its relationship with prognosis. Whilst the cutoff alcohol

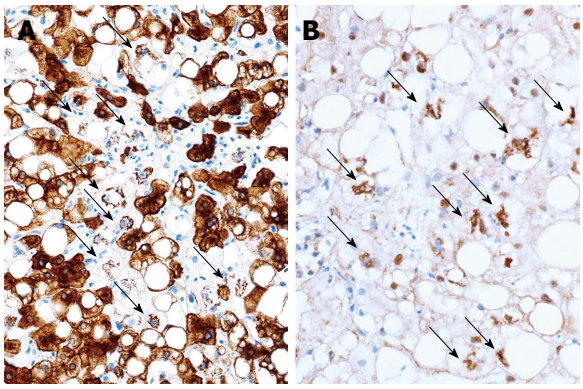

Figure 2 Typical immnohistochemical findings of nonalcoholic steatohepatitis. A: Cytokeratin (CK)18; B: Ubiquitin. Stained (brown) small aggregates are seen in ballooned hepatocytes with CK18-negative cytoplasms (arrows) [Immunoperoxidase; original magnifications, (A) × 150 and (B) × 200].

consumption ensuring "nonalcoholic" was strict (less than 20 g ethanol/d; it is almost same as the current standard, < 30 g for men and < 20 g for women[3]), they used a very loose histological criterion for diagnosing NASH. The minimal diagnostic requirements were only steatosis and inflammatory cell infiltration into lobular areas. Various clinical studies of NASH using loose diagnostic criteria similar to theirs were published thereafter[37,38]. Finally, even simple steatosis was recognized to be one aspect in the spectrum NASH and to be an ongoing change potentially progressing to more severe NASH or cirrhosis[39]. The well-known "two-hit theory" presented in the same year was generated through analogous hypothetical thinking[40]. Brunt's system[41] was established and published in such research background. They evaluated the severity of NASH using a histological score composed of inflammation grade and fibrosis stage similar to the METAVIR score[42]. The NASH severity score was used in many clinical studies, and contributed to the subsequent flowering of research on NASH. Unexpectedly, however, fairly mild fatty liver disorders were implicated in NASH cohorts by being diagnosed as grade 1 NASH. The original purpose of Brunt's system was to show a standard for evaluation of the severity of NASH. They did not seem to intend primarily to present a diagnostic criterion of NASH[43]. The expanded understanding (or misunderstanding) of NASH had rapidly spread, apart from the inventors' idea.

AFTER BRUNT AND MATTEONI (2000-PRESENT)

About ten years after the flowering of NASH/NAFLD research in Western countries, Japanese hepatologists also began to study NASH/NAFLD aggressively, due to the wide prevalence of metabolic syndrome in the beginning of the 21st century. Many NASH/NAFLD researchers in Japan understood that the minimum requirements for diagnosis of NASH were Brunt's grade 1 (without hepatocellular ballooning) and Matteoni's type 2 NAFLD. Their recognitions had been conserved even after the decision of the AASLD Single Topic Conference in 2002[2], in which hepatocellular ballooning was officially recommended as a factor in the diagnosis of NASH. They did not notice the controversy about NASH diagnostic criteria[44] and the kaleidoscope changes of the criteria in that period.

In 2005, the NAFLD activity score (NAS), which was considered as a type of modified Brunt's system, was developed and published by Kleiner *et al*[45] This also led to a simplistic recognition that a NAS of over 5 points is NASH[46], and led to further confusion in the laboratory and clinical settings.

On the other hand, Younossi *et al*[47] confirmed that hepatocellular ballooning was a predictor of liver-related death in their follow-up study, and insisted that Matteoni's classification was superior to Brunt's system or NAS. Brunt *et al*[48,49] also cautioned about the presence of cases of non-NASH NAFLD showing NAS ⩾ 5 and cases of NASH showing NAS ⩽ 4, to avoid misusage of NAS. Through their discussions and controversies[49], the diagnostic criteria of NASH/NAFLD were revised and standardized hastily. Hepatocellular ballooning finally became the most important finding in the diagnosis of NASH.

However, the difficulty in correctly identifying hepatocellular ballooning subsequently became a critical issue[50]. To overcome this problem, Bedossa *et al*[51] proposed a semi-quantitative method in which all hepatocytes with clear reticular cytoplasm were defined as ballooning and graded by the cellular size. The same research group has recently published a new diagnostic algorithm in which hepatocellular ballooning is a root node of the binary tree[52].

Examination of specific markers for ballooning has been recommended as a method to determine it objectively. Hepatocellular ballooning is a result of degeneration and fragmentation of cytoskeleton intermediate filaments, cytokeratin (CK) 8/18, and an aggregates of the degenerated CK 8/18 is a Mallory-Denk body[53]. Immunohistochemical staining for CK 18, ubiquitin and p62 can be applied to detect hepatocellular ballooning. A negative result for the presence of CK 18 in hepatocytes can be interpreted as degenerative disappearance of CK 18, and the presence of ubiquitin-, p62-, and CK 18-positive intracellular inclusions indicates aggregation of degenerated CK 18 (Figure 2). The usefulness of ubiquitin immunohistochemistry in the diagnosis of NASH was first suggested in 2000[44,54]. Recently, the significance of these special stainings has been reconfirmed[55,56].

Alternatively, controversy about such excessive weighting to hepatocellular ballooning in the diagnosis may arise. Perivenular/pericellular fibrosis (Figure 1) should be highlighted more because of its close association to hepatocellular ballooning and its potential linkage to cirrhosis[9]. The diagnostic criteria of NASH/NAFLD still remain to be improved.

FUTURE DIRECTIONS

Although the histological criteria of NASH/NAFLD have been revised during the last three decades, there has

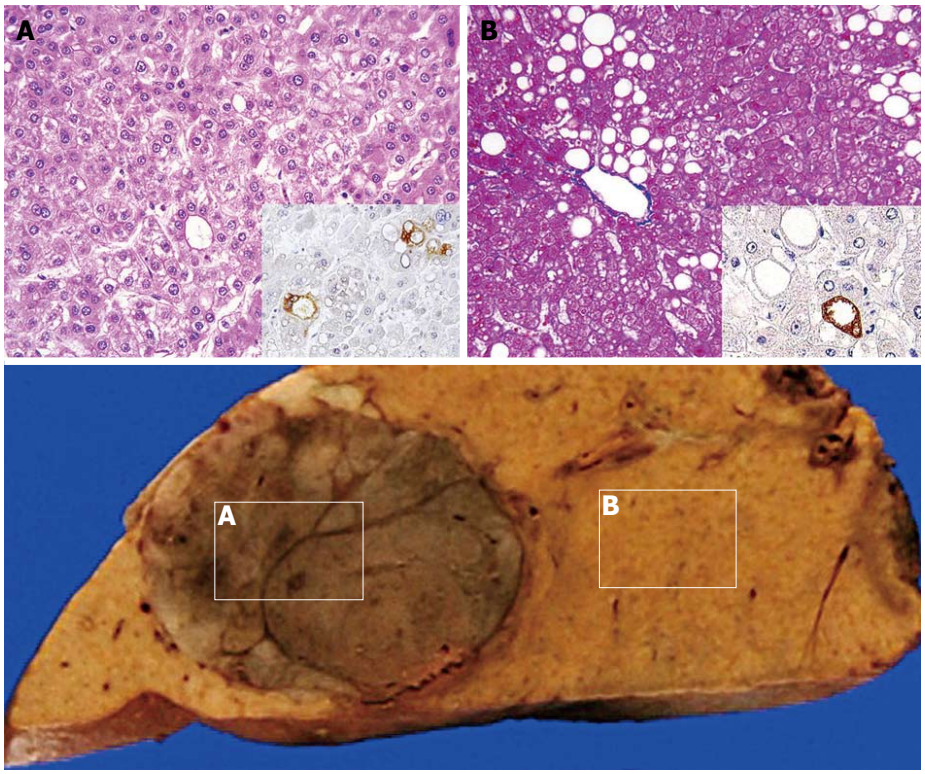

Figure 3 Case of hepatocellular carcinoma (A) associated with simple steatosis (B)[59] [upper row, histology with immunoperoxidase for a peroxidation marker (inset), original magnifications, (A) × 100 and (B) × 100; lower row, a macroscopic photo of the sample].

been an absolute premise: cirrhosis is a final advanced form of NAFLD. However, recent reports of HCC associated with NAFLD may deny the central dogma of the NASH/NAFLD concept. Surprisingly, considerable numbers of such HCCs arose from non-cirrhotic steatotic livers or even from livers with simple steatosis (Figure 3)[57-59]. Accumulation of cellular damage without major morphological changes and acceleration of cellular senescence may lead to hepatocarcinogenesis[59,60]. The facts will possibly lead to a paradigm shift in medical strategies for NAFLD.

How do we select which NAFLD patients to follow up? What is a true prognostic factor of NAFLD? Is it a histological finding? These are the ultimate themes for NAFLD researchers.

CONCLUSION

While reviewing the 30-year history of changes in the histological criteria for the diagnosis of NASH/NAFLD, the importance of hepatocellular ballooning in the diagnosis of NASH, imperfectness of the present criteria, and necessity of exploring new predictors of hepatocarcinogenesis were elucidated. Further collection of evidence is necessary to solve these problems, and pathologists will play central roles in this process.

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REVIEW

Vitamin D deficiency in chronic liver disease

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Abstract

Vitamin D is an important secosteroid hormone with known effect on calcium homeostasis, but recently there is increasing recognition that vitamin D also is involved in cell proliferation and differentiation, has immunomodulatory and anti-inflammatory properties. Vitamin D deficiency has been frequently reported in many causes of chronic liver disease and has been associated with the development and evolution of non-alcoholic fatty liver disease (NAFLD) and chronic hepatitis C (CHC) virus infection. The role of vitamin D in the pathogenesis of NAFLD and CHC is not completely known, but it seems that the involvement of vitamin D in the activation and regulation of both innate and adaptive immune systems and its antiproliferative effect may explain its importance in these liver diseases. Published studies provide evidence for routine screening for hypovitaminosis D in patients with liver disease. Further prospectives studies demonstrating the impact of vitamin D replacement in NAFLD and CHC are required.

Key words: Cholecalciferol; Vitamin D; Hepatitis C; Liver fibrosis; Liver disease; Interferon; Sustained virological response; Nonalcoholic fatty liver disease; Nonalcoholic steatohepatitis

Core tip: (Vitamin D and liver disease) vitamin D deficiency has been frequently reported in many causes of chronic liver disease and has been associated with the development and evolution of non-alcoholic fatty liver disease (NAFLD) and chronic hepatitis C (CHC) virus infection. The role of vitamin D in the pathogenesis of NAFLD and CHC is not completely known, but it seems that the involvement of vitamin D in the activation and regulation of both innate and adaptive immune systems and its antiproliferative effect may explain its importance in these liver diseases.

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INTRODUCTION

Vitamin D insufficiency and deficiency are prevalent in almost half the healthy population of developed countries[1]. Most experts define vitamin D insufficiency as a 25(OH)D level below 75 nmol/L (30 ng/mL) and deficiency as levels below 50 nmol/L (20 ng/mL). It is estimated that one billion people suffer from deficiency or insufficiency of vitamin D[2]. In the United States, between 25% and 50% of the adult population has vitamin D deficiency[3]. In patients with chronic liver diseases, the prevalence of vitamin D deficits is much higher and practically universal[4]. Up to 93% of patients with chronic liver disease have insufficient vitamin D levels, and almost one-third of these show severe deficiency[5].

The outcome of vitamin D deficiency in terms of osteoporosis, osteomalacia and increased fracture risk is well known[6,7]. Furthermore, the association between vitamin D deficiency and the development of infections, cardiovascular, autoimmune and degenerative diseases and several types of cancer (colon, prostate and breast cancer) has also been reported[8]. Vitamin D is important

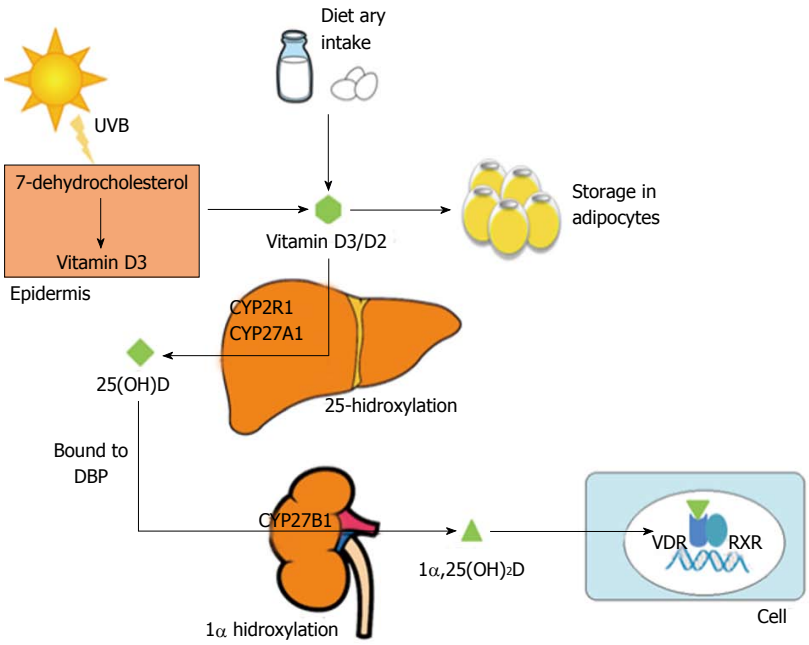

Figure 1 Vitamin D synthesis. VDR: Vitamin D receptor; DBP: Vitamin D-binding protein; UVB: Ultraviolet radiation; RXR: Retinoid X receptor.

in calcium homeostasis and has also been implicated in the mechanisms of cellular proliferation, differentiation and immunomodulation[9]. These effects are noted in the pathogenesis and treatment of many chronic liver diseases. In this review, we will focus on vitamin D functions involved in the development of chronic liver disease and on the relationship between vitamin D deficiency and the two main causes of chronic liver disease: chronic hepatitis C (CHC) virus infection and non-alcoholic fatty liver disease (NAFLD).

An evidence-based approach was used for this review. MEDLINE search was performed to September 2014 using the following MeSH terms: liver diseases, vitamin D, cholecalciferol, hepatitis C, Chronic, nonalcoholic fatty liver disease. Searches were limited to English language articles. References of suitable articles were searched for other appropriate articles.

VITAMIN D SYNTHESIS

Under normal conditions, biogenesis from epidermal cells is the main source of vitamin D. In the skin, ultraviolet radiation from sun exposure transforms 7-dehydrocholesterol, a metabolite of cholesterol, into pre-vitamin D_3, which is transformed into vitamin D_3 (cholecalciferol). A small portion of vitamin D comes from dietary sources, such as milk and eggs, in the form of vitamin D_2 (ergocalciferol) and D_3 that is absorbed in the intestine by biliary acids[1,10]. Vitamin D synthesized from skin and from dietary sources may be stored in the adipocytes, or it may undergo hepatic 25-hydroxylation. This latter process is mediated by isoforms of the P450 cytochrome (CYP2R1, CYP27A1), the 25-hydroxylases, which produce 25-hydroxyvitamin D [25(OH)D] or calcidiol. The metabolite 25(OH)D, most abundant in blood, is an inactive form of vitamin D. It has a half-life of 2-3 wk and is a useful measure of vitamin D levels because it reflects the total amount of vitamin D from dietary sources, sun exposure and conversion from fatty deposits of the liver, and its concentration in plasma is the most reliable indicator of vitamin D status[11]. This vitamin D metabolite, like others, is a low-solubility lipophilic molecule that moves through the bloodstream attached to plasmatic proteins, the most prevalent of which is vitamin D-binding protein (DBP), also known as Gc. Up to 88% of serum 25(OH)D is attached to a DBP, a protein synthesized mainly in the liver that has anti-inflammatory and immunomodulating functions independent of its role as a vitamin D transporter[12,13]. 25(OH)D is hydroxylated in the proximal tubules of the kidney by 1α-hydroxylase (CYP27B1) that form $1\alpha,25(OH)_2D$ or calcitriol, the most biologically active and powerful metabolite of vitamin D[1]. CYP27B1 activity has been observed in the kidney and other tissues, including the liver, fat tissue and the cells of the innate immune system[14]. Finally, 24-hydroxylase, which is most abundant in the intestine and the kidney, catabolizes the calcitriol into an inactive metabolite that is excreted in bile[15] (Figure 1).

$1\alpha,25(OH)_2D$ has a half-life of 4 h. It is transported *via* attachment to plasmatic proteins such as DBP and, as mentioned previously, conducts most of the biological effects of vitamin D by directly and indirectly controlling the expression of over 200 genes linked to angiogenesis, apoptosis, proliferation, differentiation and immunomodulation[1,16,17]. The biological effects of vitamin D are mediated by binding to the vitamin D receptor (VDR), belongs to the superfamily of nuclear steroid hormone receptors, which is expressed in more than 30 tissues, including the liver, the pancreatic islet cells, the epithelial cells of the gastrointestinal tract and the immune system

cells[18]. Hence, vitamin D deficiency may be involved in several processes, such as cancer, diabetes mellitus (DM) and cardiovascular and autoimmune diseases[19-26]. Furthermore, the immune system cells, including macrophages, dendritic cells, and T and B lymphocytes, express CYP27A1 or CYP27B1 enzymes and thus can metabolize 25(OH)D to calcitriol. Calcitriol will then have an autocrine or paracrine function[19,20]. Vitamin D favors the innate response of the immune system and has a "self-regulatory" effect by limiting the adaptive response. On one hand, it stimulates the synthesis of antimicrobial peptides (cathelicidin and beta-defensin) and the chemotaxis and phagocytosis of the macrophages. On the other hand, it decreases the expression of class Ⅱ complex molecules, co-stimulating molecules and the synthesis of Th1, Th2 and Th17 cytokines[19,20]. Finally, in addition to acting as a transcription factor, VDR seems to induce fast non-genomic responses by activating cellular signaling pathways. In this sense, has been shown presence of VDR in plasma membranes of intestinal, lung, kidney, muscle cells and osteoblasts, where it efficiently binds $1\alpha,25(OH)_2D$[16,27,28].

REGULATORY MECHANISMS OF VITAMIN D SYNTHESIS

The synthesis process of vitamin D includes regulatory mechanisms in each step, as follows: (1) in the skin, excess of vitamin D_3 is destroyed by sunlight, thus preventing vitamin D_3 intoxication from excessive sun exposure[29]; (2) the 25-hydroxylation of vitamin D is under-regulated. The levels of 25(OH)D increase according to the intake of vitamin D; thus, plasmatic levels of 25(OH)D are used to regulate vitamin D status; (3) in contrast, 1α-hydroxylase is highly regulated. Different factors are involved in its activity and expression, including serum calcium and phosphate, parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23). An elevated calcium serum concentration suppresses 1α-hydroxylase directly and indirectly by decreasing the PTH levels[30]; elevated plasmatic phosphate also decreases the expression and activity of 1α-hydroxylase through a mechanism that is not yet understood. This increase in serum phosphate seems to trigger an increase of FGF23 that inhibits $1\alpha,25(OH)_2D$ synthesis[31]. Furthermore, the synthesis and degradation of $1\alpha,25(OH)_2D$ is also controlled by local factors such as cytokines and growth factors, although this local production has no effect on the blood levels[32,33]. In the case of the macrophages, the expression of CYP27B1 and synthesis of $1\alpha,25(OH)_2D$ are induced by inflammatory cytokines, such as interferon (IFN)γ, and by toll-like receptor (TLRs) ligands, such as the lipopolysaccharide (LPS); (4) the 25-hydroxyvitamin D-24-hydroxylase (CYP24A1) catabolizes $1\alpha,25(OH)_2D$ to calcitroic acid, a biologically inactive bile-excreted metabolite[15]. The activity and expression of this enzyme, which is most abundant in intestine and kidney, is controlled by the levels of $1\alpha,25(OH)_2D$, phosphate and PTH[34,35]; (5) the DBP protein may buffer the levels of free vitamin D which is correlated with the levels of active vitamin D, this prevents intoxication[36]. Additionally, DBP prevents catabolism and excretion of the hormone. The DBP levels decrease in liver disease, nephrotic syndrome and malnutrition; despite this modification, the concentration of $1\alpha,25(OH)_2D$ remains constant; and (6) $1\alpha,25(OH)_2D$ controls its own synthesis not only through the increase of CYP24A1 expression, as mentioned above, but also by directly or indirectly inhibiting CYP27B1 expression and providing a negative feedback pathway.

Therefore, we can conclude that multiple factors regulate vitamin D metabolism. The intake of vitamin D through diet or sun exposure is only one of many variables that determine its activity, another of these variables are DBP levels, the local synthesis of $1\alpha,25(OH)_2D$ (the autocrine or paracrine effect) and VDR expression.

VITAMIN D AND CHRONIC LIVER DISEASE

As discussed previously, vitamin D plays an important role in reducing the risk of chronic diseases, including DM type 2, several types of cancer, and cardiovascular, autoimmune and infectious diseases. This role most likely results from the local production of $1\alpha,25(OH)_2D$ and its autocrine and paracrine actions in cellular proliferation and differentiation, apoptosis, insulin and renin secretion and interleukin (IL) and bactericidal protein production[1,16,17,19-23]. These effects may also be relevant in the pathogenesis of chronic liver diseases.

Vitamin D deficiency is extremely common in chronic liver disease patients. Up to 93% of these patients have some degree of vitamin insufficiency[4,5]. Even patients with mild liver disease are affected, although liver cirrhosis patients more commonly suffer from severe deficiency.

Several studies in general populations have shown that low levels of 25(OH)D significantly increase the risk of mortality from all causes, including cardiovascular diseases[37,38]. Regarding patients with chronic liver disease of varying etiologies, vitamin D deficiency has been associated with increased mortality[39,40], bacterial infections[41], portal hypertension complications[42] and fibrosis severity[43,44]. However, because the liver plays an important role in the metabolism and pleiotropic functions of vitamin D, the question is whether vitamin D deficiency is a consequence of liver disease or a contributor to the liver dysfunction.

Severe liver disease decreases vitamin D hydroxylation and albumin and DBP production, all of which are linked to low levels of 25(OH)D. Nevertheless, the vitamin D deficiency in chronic liver disease is only partly the result of a synthesis dysfunction of the liver, as evidenced by the fact that vitamin D deficiency is highly prevalent in non-cirrhotic patients[4]. The levels of 25(OH)D in cirrhotic patients normalize after vitamin D treatment, which indicates that the 25-hydroxylation is pre-

served[45,46]; and although DBP is moderately decreased in cirrhosis[47], vitamin D metabolites require only 5% of the DBP binding sites[48], indicating that liver dysfunction must be severe to decrease the DBP levels and contribute to vitamin D deficiency. Therefore, vitamin D deficiency in chronic liver disease requires several causes in addition to those mentioned above, including inadequate sun exposure, insufficient food intake, steroid use, jaundice-related deterioration of vitamin synthesis on the skin and decreased vitamin D absorption caused by intestinal edema secondary to portal hypertension or to cholestasis-induced bile salt disruption.

The observed association between vitamin D and liver disease is insufficient to establish a causal effect between vitamin D deficiency and the severity of chronic liver disease. Recent systematic and umbrella reviews has cast doubt on any causal link between vitamin D deficiency and non-skeletal health outcomes, suggesting that vitamin D deficiency is a marker of ill-health, rather than an important factor implicated in the pathogenesis of disease[49]. However, there is growing evidence that vitamin D is involved in the decrease of inflammation and fibrosis[43,50,51]. Proinflammatory signals in monocytes and macrophages may regulate the local metabolism of vitamin D, auto-inducing the expression of CYP27B1 and the local production of $1\alpha,25(OH)_2D$, and thus controlling the excessive inflammatory response[33,52]. Almost 90% of the tissue macrophages are in the liver[53], which suggests that the liver production of active vitamin D is affected during inflammatory diseases of the liver. Furthermore, VDR is expressed in both macrophages and other non-parenchymal cells and biliary epithelial cells[54]. After activation, these cells increases the expression of cathelicidin, an antimicrobial peptide with anti-endotoxin activity[55], and inhibits the synthesis of biliary acids, thus protecting the hepatocytes from these acids[56,57]. Therefore, the relationship between vitamin D and hepatic physiopathology may result from signaling disruptions in non-parenchymal liver cells or extrahepatic cells[58].

It is important to mention that, together with diet intake and sun exposure, genetic factors substantially contribute to variations in 25(OH)D levels[59,60]. Several simple nucleotide polymorphisms of genes involved in the metabolism of VDR and vitamin D, such as DHCR7 (encode the 7-dehydrocholesterol reductase enzyme), CYP2R1, CYP24A1 and GC (encode DBP), have been strongly linked with the serum levels of 25(OH)D and its efficacy[59-62]. A recent study community-dwelling black Americans, as compared with whites, had low levels of total 25(OH)D and DBP, resulting in similar concentrations of estimated bioavailable 25(OH)D. Racial differences in the prevalence of common genetic polymorphisms provide a likely explanation for this observation[63]. Therefore, such genetic variations may be associated with the severity of chronic liver disease, and several polymorphisms of the *VDR* gene associated with primary biliary cirrhosis, autoimmune hepatitis, CHC and hepatocellular carcinoma have been identified[64-69].

The available data suggest that vitamin D supplements could be beneficial in terms of morbimortality[70,71]. Most experts consider of at least 75 nmol/L (30 ng/mL) as the most advantageous 25(OH)D level for reducing the risk of fractures, prevention of cancer and the risk of hypertension, and between 90-120 nmol/L (36-48 ng/mL) as the most optimal level[71]. In fact, a recent meta-analysis that included 73 cohort studies (849412 participants) and 22 controlled and randomized studies with over 30716 participants showed that vitamin D_3 supplements significantly reduced mortality from any cause among older adults[72]. Few published prospective studies have examined the effects of supplements in chronic liver disease, and the results to date are contradictory, most likely because of issues with study designs, the quantity of vitamin D administered, the pre- or post-treatment measurements used and the presence of genetic polymorphisms that influence the biological activity of vitamin D. Nonetheless, vitamin D supplements are currently recommended to decrease the skeletal effects of vitamin D deficiency. In fact, the latest recommendation suggest that a 25(OH)D level over 20 ng/mL is sufficient to meet the vitamin D requirement[73]. However, the Endocrine Society Clinical Practice Guideline (ESCPG) suggested that vitamin D requirements may be greater for sick patients than for healthy individuals and blood level above 30 ng/mL may have additional health benefits in reducing the risk of various disease conditions[74]. In addition, the ESCPG suggest that 25(OH)D should be measured in chronic liver disease patients to identify those with levels under 20 ng/mL who would benefit from vitamin D supplements to reduce the risk of bone fracture[74]. Similarly, the guidelines of the European Association for the Study of the Liver recommend calcium (1000-1200 mg/d) and vitamin D (400-800 UI/d) supplements for cholestatic liver disease patients, although supplement use is supported by limited clinical data[75]. In fact, despite the frequency of vitamin D deficiency in liver disease patients, their calcium and PTH serum concentration levels are normal, which contradicts the possibility that regulatory mechanism of calcium metabolism is affected[76,77]. Our group has confirmed these results in cirrhotic patients of different etiologies; these patients showed vitamin D deficiencies[78] but had free vitamin D levels similar to those of healthy subjects (unpublished data). Consequently, the unaffected free vitamin D may be involved in the lack of correlation between the levels of 25(OH)D and calcium and PTH and may maintain calcium homeostasis without causing secondary hyperparathyroidism[79]. For this reason, several authors indicate that the levels of total and free 25(OH)D should be measured to identify the vitamin D status in chronic liver disease patients[76]. Nonetheless, these patients have a high prevalence of bone mass loss that can be explained by the previous data of vitamin D deficiency and by other interfering factors, such as the increase in pro-inflammatory cytokines[80-82], hypogonadism[83], elevated bilirubin levels[84] and steroid treatment[85].

VITAMIN D FUNCTIONS AND THEIR IMPLICATIONS IN LIVER DISEASES

Vitamin D maintains the normal skeletal architecture and plays roles in the cardiovascular[86,87] and nervous systems[88,89] and cellular proliferation and differentiation[90,91]. Furthermore, vitamin D may be relevant in the physiopathology of chronic liver diseases because of its effect on the immune system and its anti-fibrotic effect[51,92,93].

Several research lines suggest that vitamin D has beneficial effects in liver diseases by activating and regulating innate and adaptive immunity.Vitamin D increases innate immunity[23], stimulating the mechanisms associated with the elimination of pathogen agents through the secretion of antibacterial proteins, such as cathelicidin and beta-defensin, and favoring chemotaxis and macrophage phagocytosis[19,20,94,95]. An excessive immune response can cause tissue damage; in this sense, vitamin D promotes an adequate innate immune response by regulating the expression of several TLRs and by decreasing the production of proinflammatory cytokines[52]. An inverse relationship between vitamin D levels and the expression of TLR2, TLR4 and TLR9 in monocytes has been observed, as has a decrease in the expression of these innate immunity receptors after the administration of $1\alpha,25(OH)_2D$[52,96,97]. These three TLRs are primarily related to the inflammation and fibrosis of the liver. A high-fat diet, alcohol consumption and structural changes in the intestinal mucosa resulting from chronic liver diseases (*e.g.*, the loss of epithelial attachment, vascular congestion, defects of the mucosal immune system) alter the permeability of the mucosa, promoting an increase in intestinal bacteria translocation[98-100] and bacterial products, such as LPS, through the bloodstream; there, these bacteria bond to the TLRs, mainly TLR4, that are present such immune cells as hepatocytes, biliary epithelial cells, dendritic cells and hepatic stellate cells, triggering the synthesis of proinflammatory cytokines and fibrogenesis that ultimately result in liver damage[98,101]. However, vitamin D is involved not only in the regulation of TLR expression but also in intestinal permeability; it plays a role in intestine epithelial cell differentiation and in improving cell bonding[102,103], thus decreasing the bacterial products in the liver.

Regarding adaptive immunity, vitamin D seems to control an excessive immune response by decreasing the expression of class II HLA complex molecules and co-stimulator molecules and by modulating the T cell response[19,20,104]. The activation of naïve T cells has been shown to be vitamin D-dependent[105]; furthermore, it inhibits the development of Th1 (IL-2 and interferon-gamma proinflammatory cytokine producers) and Th9 and increases the number of Th2 cells (IL-4, 5 and 10 anti-inflammatory cytokine producers), thus affecting the polarization of T helper cells[106-108]. Additionally, $1\alpha,25(OH)_2D$ prevents the development of Th17 cells by inhibiting IL-6 and IL-23 production from the dendritic cells, and it induces the differentiation and expansion of regulatory T cells that secrete the anti-inflammatory cytokines IL-10 and transforming growth factor beta (TGF-β)[107,109,110]. This ability to modulate the adaptive immune system may explain the association between vitamin D deficiency and the risk of autoimmune diseases and liver damage.

Moreover, *in vitro* and *in vivo* studies of mouse models with liver fibrosis have reported that vitamin D has an anti-fibrotic effect due to ability to affect the pathological process of liver fibrosis at several stages, such as: inhibition of injury trigger, suppression of hepatic stellate cells activation and proliferation, reduction in accumulation of extracellular matrix and even degradation of collagen metalloproteinases activation and tissue inhibitor matrix metalloproteinases (TIMPs) inhibition[92,93]. Moreover, Ding *et al*[111] revealed an intersecting VDR/SMAD genomic circuit that regulates hepatic fibrogenesis and define a role for VDR as an endocrine checkpoint to modulate the wound-healing response in liver and VDR ligands as potential therapy for liver fibrosis[111]. In this regard, a recent study in mice showed that the active metabolite of vitamin D-$1\alpha,25(OH)_2D$ may prevent liver fibrosis in the *in-vivo* model. However, it cannot ameloriate establish cirrhosis in an animal model[112].

VITAMIN D AND CHRONIC HEPATITIS C VIRUS INFECTION

Epidemiological studies show that vitamin D deficiency may increase the risk of acquiring viral infections such as influenza, human immunodeficiency virus and respiratory infections[113]. Chronic hepatitis C virus (HCV) infection is one of the main causes of chronic liver disease; it is estimated to affect 130 to 150 million people worldwide, a significant number of whom also develop cirrhosis and hepatic cancer[114]. A high percentage of these patients (46% to 92%) have low vitamin D levels[50,115-117], and more than 25% suffer from severe deficiency[50,115,117]. It has been hypothesized that the high incidence of vitamin D deficiency in these patients may be caused by HCV's effect on direct or indirect 25-hydroxylation through cytokine induction or oxidative stress[118,119] and that the virus may suppress 25(OH)D levels due to a disruption in lipid metabolism; as shown a recent study where HCV decreases the production of 7-dehydrocholesterol, the endogenous precursor of vitamin D[120].

As discussed previously, vitamin D inhibits fibrosis and modulates the innate and adaptive immune response, increases the production of antimicrobial peptides and inhibits proinflammatory cytokines. The anti-inflammatory action of vitamin D[19,20,50,94,95,104,106-110] can explain the improved therapeutic results of IFN and ribavirin (RBV) after the administration of vitamin D supplements[121-123], as some data indicate that proinflammatory cytokines and chemokines promote the persistence of HCV[124]. In this respect, a low Th1/Th2 ratio is an independent sustained viral response (SVR) factor in the treatment of the HCV genotype 1[125], and $1\alpha,25(OH)_2D$ favors Th2 in this balance, as mentioned previously[108]. Furthermore, several

Table 1 Studies regarding vitamin D and hepatitis C virus

Ref.	Year	Design	*n*	HCV genotype	Vitamin D deficiency	Outcome	*P*
Petta *et al*[50]	2010	Cohorts	197	1	73%	Vitamin D levels (ng/mL): SVR: 26.6 No SVR: 23.7	0.05
Bitetto *et al*[121]	2011	Cohorts	42	1 and no 1	Not stated	SVR according to the vitamin D levels (ng/mL): ⩽ 10 ng/mL: 10% > 10 and ⩽ 20 ng/mL: 30% > 20 ng/mL: 50%	< 0.05
Bitetto *et al*[136]	2011	Cohorts	211	1-5	46.4%	SVR according to the vitamin D levels (ng/mL): ⩽ 10 ng/mL: 50% > 10 and ⩽ 20 ng/mL: 60.9% > 20 ng/mL: 69%	0.038
Lange *et al*[115]	2011	Cohorts	468	1-3	66%	SVR (genotype 2/3): Vitamin D deficit (< 10 ng/mL): 50% Without deficiency: 81% SVR (genotype 1) Vitamin D deficit: 60% Without deficiency: 54%	< 0.0001 0.45
Nseir *et al*[133]	2011	Cohorts	80	1	Not stated	Vitamin D levels (ng/mL): SVR: 42.1 No SVR: 27.3	< 0.001
Jazwinski *et al*[134]	2011	Cohorts	82	1	Not stated	Vitamin D levels (ng/mL): SVR: 23.3 No SVR: 19.3	0.82
Abu-Mouch *et al*[123]	2011	Randomized prospective	72	1	59% (with vitamin D supplementation) 60% (control group)	SVR: With vitamin D: 86% Control group: 42%	< 0.001
Nimer *et al*[122]	2012	Randomized prospective	50	2-3	60% (with vitamin D) 50% (control group)	SVR: With vitamin D: 95% Control group: 77%	< 0.001
Lange *et al*[116]	2012	Cohorts	269	1-4	74%	No significant association between SVR and 25(OH)D serum levels	0.13
Kitson *et al*[137]	2013	Cohorts	274	1	48%	Vitamin D levels (ng/mL): SVR: 76.6 No SVR: 84.7	0.03
Esmat *et al*[140]	2014	Randomized prospective	101	4	95%	SVR: With vitamin D: 44% Control group: 68.6%	0.22
Yokoyama *et al*[142]	2014	Randomized prospective	84	1b	Not stated	SVR: With vitamin D: 64.3% Control group: 50%	0.19
Grammatikos *et al*[138]	2014	Cohorts	398	1	Not stated	Vitamin D levels (ng/mL) SVR: 15.8 No SVR: 17.6	0.09

HCV: Hepatitis C virus; SVR: Sustained viral response.

in-vitro studies have considered vitamin D a direct HCV antiviral agent[126-128]. Gal-Tanamy *et al*[127] showed that vitamin D increases VDR expression and inhibits HCV replication in human hepatocytes by inducing the expression of IFN beta and the IFN-stimulated gene (*MxA*) with different antiviral properties, thus producing a synergic effect with antiviral treatment[127]. In the same study, vitamin D or calcitriol added to the antiviral treatment had a synergic effect in the inhibition of HCV. In addition, in recent clinical studies have described an association between VDR polymorphisms on the response to IFN/RBV therapy in CHC[129,130].

The relevance of vitamin D in CHC has been reported in numerous studies that associated vitamin D deficiency with a greater degree of necrosis and fibrosis[40,50,68,131,132] and with a lower likelihood of a SVR to IFN-based therapies[50,115,121,123,133-135]. In fact, all of the patients who showed severe vitamin D deficiency had hardly any SVR, while 50% of those with normal levels or almost normal levels had SVR[50,121,123,136]. However other studies failed to find ant relationship between baseline vitamin D level and SVR and fibrosis[116,137-140] (Table 1). In addition, conflicting conclusions have been reached in two recent meta-analysis[130,141]. This may be due to limitations of the studies included: (1) the small number of patient; (2) majority had a cross-sectional studies that are subject to bias due to the possibility of reverse causation; (3) lack of vitamin D level assessment during therapy;

and (4) characteristic of vitamin D assessment (seasonality, cut off values, methodology of vitamin D determination, ethnicity). In contrast, vitamin D has been shown to increase the probability of SVR when it is added to the antiviral treatment[121-123,142] (Table 1). Thus, futher clinical investigation on the effect of vitamin D supplementation in treating CHC are needed to confirm this item.

Furthermore, Bitteto *et al*[136] provided additional information in their study of the rs12979860 C/T polymorphism of IL28B. In their study, vitamin D levels were complementary to the rs12979860 C/T polymorphism of the IL28B for predicting SVR in CHC patients infected with difficult-to-treat genotypes (1, 4, 5). Another polymorphism, the CYP27B1-1260 polymorphism is also known to decrease the intracellular concentration of calcitriol in mononuclear cells and T lymphocytes[134] and is a known cofactor in immune response disruption in these cells. In fact, the study by Lange and colleagues confirms the lack of SVR in patients infected with the HCV 1, 2 and 3 genotypes who have this polymorphism[115]. This study also hypothesized that genotype 3 patients had low 25(OH)D levels, in contrast with previously published data[50,136]. We should, however, note that the definition of vitamin D deficiency differed among the three studies, a factor that should be considered when interpreting these results.

Vitamin D also favors the HCV response by improving the sensitivity to insulin[143-145]. Insulin resistance (IR) is considered one of the most important factors in predicting HCV patients' response to IFN and RBV[146], and vitamin D is known to prevent DM type 2[144]. As β-pancreatic cells contain VDR, vitamin D deficiency may alter the balance between the intra- and extracellular calcium and interfere with insulin release[147].

Therefore, in theory, vitamin D deficiency may be linked to a lack of response to anti-viral treatment, while vitamin D supplementation may potentiate SVR.

VITAMIN D AND NAFLD

NAFLD is a pathological clinical entity that includes a broad spectrum of liver conditions from steatosis to nonalcoholic steatohepatitis (NASH) and cirrhosis[148] and NAFLD is one of the main causes of chronic liver disease in developed countries, affecting 20% to 30% of the population[149,150]. Some NAFLD patients develop NASH and cirrhosis, while most others do not experience disease progression; however, the reason for these differences in progression are not known. NAFLD is generally related to at least one metabolic syndrome characteristic; in fact, liver conditions are considered part of the syndrome, and although their pathogenesis is not yet known, IR is a key factor in its development[151,152]. Several studies show a negative correlation between vitamin D levels and obesity, glucose intolerance, IR, metabolic syndrome and body mass index (BMI)[24-26,153-155]. Furthermore, vitamin D deficiency stimulates PTH, which has been linked to IR and an increase in the acute-phase reactant[156]. In support of this hypothesis, some studies show that vitamin D administration improves insulin secretion[145,157-160] and that its use decreases IR in patients with end-stage renal disease[161]. Moreover, VDR polymorphisms have been associated with IR and have an effect on insulin secretion and on the fasting glucose concentration[162]. Additionally, previous studies have shown that VDR knock-out mice developed hepatic steatosis[163]. Finally, studies have shown that vitamin D administration in mice activates the fibroblastic intestinal growth factor 15 (FGF15) (human ortholog FGF19). This intestinal hormone prevents IR and high-fat diet-induced obesity by inhibiting CYP7A1, an essential enzyme in the physiopathology of liver dyslipidemia[164]. This evidence suggests that vitamin D is linked to the development of NAFLD *via* its role in glucose metabolism by accelerating the conversion of proinsulin to insulin, while vitamin D deficiency has been associated with pancreatic β cell dysfunction and a greater prevalence of type 2 DM[153,164-167].

As in the case of CHC, vitamin D levels are lower in patients with NAFLD compared with healthy controls[43,167-174]. In addition, vitamin D deficiency in obese patients has been attributed to the accumulation of the vitamin D in adipose tissue[175-177]. Furthemore, vitamin D levels are inversely correlated with the severity of steatosis, necroinflammation and fibrosis independent of age gender, BMI, Homeostatic Model Assesment of IR score and presence of metabolic syndrome[43,168,178]. In a recent clinical study of adults with NAFLD, Targher *et al*[43] showed that the vitamin D levels had an effect on the development of hepatic steatosis and in the severity of the histological lesion. In fact, their hypothesis stated that patients with greater inflammation and fibrosis had lower vitamin D levels independent of other components of the metabolic syndrome. This observation was later confirmed in pediatric patients[179,180] (Table 2). Still, an association between vitamin D and NAFLD has been demonstrated that is independent of BMI or IR and metabolic syndrome[43,157,162]. Although causal conclusions are difficult to obtain from these studies, their results suggest that vitamin D deficiency plays a role in the development and progression of fatty liver, especially in terms of its anti-inflammatory potential. In fact, vitamin D reduces the risk for NAFLD in healthy men[181] and attenuates high fat diet-induced hepatic steatosis in rats by modulating lipid metabolism[182].

Vitamin D deficiency has been linked to a systemic increase in inflammation markers[183,184], and systemic inflammation may play a central role in the pathogenesis and progression of NAFLD[185,186]. Increases in visceral adiposity promote the release of fatty acids and proinflammatory cytokines and activate inflammation pathways in the liver, prompting proinflammatory cytokine secretion that leads to liver damage[187]. Moreover, the obesity promotes the onset of NAFLD due to increased hepatic lipid synthesis secondary to excess free fatty acids; subsequent association with oxidative stress on mitochondrial and with the increase of proinflammatory

Table 2 Studies regarding vitamin D and non-alcoholic fatty liver disease

Ref.	Year	Design	*n*	NAFLD diagnosis	Vitamin D levels (ng/mL)	*P*
Targher *et al*[43]	2007	Cohorts prospective	120	Liver biopsy	Controls (60): 29.8 ± 6 Steatosis (10): 23.72 ± 8 NASH (50): 14.8 ± 9.2	0.001
Manco *et al*[179]	2010	Cohorts prospective	64	Liver US	Without necroinflamation: 26.1 ± 10 With necroinflamation: 19.9 ± 9.8 Without fibrosis: 27.7 ± 10.3 With fibrosis: 17.1 ± 7.4	0.16 < 0.001
Barchetra *et al*[168]	2011	Cohorts prospective	262	Liver US	Without NAFLD (100): 20.5 ± 9.7 NAFLD (162): 14.8 ± 9.2	< 0.001
Jablonski *et al*[169]	2013	Cohorts retrospective	1214	Liver US	Controls (607): 34 ± 8 NAFLD (607): 30 ± 7	< 0.001
Kasapoglu *et al*[171]	2013	Cohorts prospective	613	Liver US	Controls (275): 26,4 ± 9.8 NAFLD stage 1 (133): 20 ± 9.2 NAFLD stage 2 (106): 13.3 ± 6.7 NAFLD stage 3 (99): 8.8 ± 7.4	< 0.05
Black *et al*[170]	2014	Cohorts prospective	994	Liver US	Without NAFLD (838): 30.8 ± 9.6 NAFLD (156): 26.8 ± 8.8	< 0.001
Yildiz *et al*[174]	2014	Cohorts prospective	101	Liver US	Without NAFLD (43): 16.4 (IQR 12.4-24.8) NAFLD grade 1 (41): 14.2 (IQR 9.5-21.2) NAFLD grade 2 (17): 11.5 (IQR 7.5-16.7)	0.005
Dasarathy *et al*[178]	2014	Cohorts prospective	148	Liver biopsy	Controls (39): 35.7 ± 6 Steatosis (67): 25 ± 11.3 NASH (81): 18.1 ± 8.4	< 0.01
Nobili *et al*[180]	2014	Cohorts prospective	73	Liver biopsy	NASH (49) was associated with lower VD levels, *i.e.*, -9.0 pg/mL when compared with that in children without NASH (24)	< 0.001
Küçükazman *et al*[173]	2014	Cohorts prospective	211	Liver US	Without NAFLD (57): 20 ± 13.6 NAFLD (154): 12.3 ± 8.9	< 0.001

US: Ultrasonography; IQR: Interquartile range; NAFLD: Non-alcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis.

cytokines can definitely trigger a progression of steatosis to NASH and cirrhosis[188]. Studies *in vivo* and *in vitro* have clearly documented that steatosis reduces oxidative activity controlled by cytochrome P450[189]. These inflammatory processes may be blocked by increasing the levels of 25(OH)D, and the development and progression of NAFLD may stop. In fact, vitamin D supplements have been shown to decrease inflammation markers[190-193] and increase anti-inflammatory cytokines[190]. It is known that vitamin D's effects in the liver are not only exerted on the hepatocytes, given that these cells express very little VDR mRNA. In contrast, sinusoidal cells, Kupffer cells, hepatic stellate cells and immune system cells express VDR mRNA that is functionally active. Therefore, vitamin D deficiency may affect the activity/expression of macrophages, dendritic cells and T and B lymphocytes by favoring oxidative stress and the production of proinflammatory cytokines that lead to subclinical inflammation[18,19]. Furthermore, fibrosis is induced by TGF-β secretion that results from the increased secretion of the matrix metalloproteinase 9 inhibitor (TIMP-1)[194]. In fact, cell cultures show that vitamin D has an anti-inflammatory and an antifibrinolytic effect on hepatic stellate cells. Finally, animal models show that more severe histological lesions of NAFLD are associated with higher levels of mRNA of TLR2, 4 and 9, proinflammatory cytokines and oxidative stress markers in rats with a high-fat diet and deficient in vitamin D[195]. A recent study of experimentally NAFLD-induced rats showed that ultraviolet light exposure decreased hepatic stellate cell activity and TGF-β synthesis and stimulated the production of apolipoprotein E and adiponectin. Together, these findings translate into a beneficial effect on NAFLD, and a decrease in IR, steatosis, apoptosis, inflammation and intrahepatic fibrosis was hypothesized[196]. Thus, given the above-mentioned findings, we can conclude that extrahepatic signaling affects fibrosis and inflammation[187] and that the vitamin D-VDR axis may play a role in the initiation and progression of NAFLD.

Therefore, although the mechanisms of vitamin D's control over hepatic lipid homeostasis and its link with inflammation are not fully known, recent research lines provide a more comprehensive understanding of its immune modulation capacity and of new therapeutic interventions for NAFLD.

CONCLUSION

The pleiotropic effects of vitamin D indicate a relationship between its deficiency and numerous chronic diseases, such as DM, cardiovascular, autoimmune and infectious diseases, several types of cancer and chronic liver diseases. In the case of chronic liver diseases, vitamin D seems to modulate the innate and adaptive immune system, which explains the association. Specifically, vitamin D deficiency has been associated with a greater risk of portal hypertension complications, mortality and increased histological severity in NAFLD and CHC, and

a lower probability of viral response to HCV treatment with IFN based therapies. In fact, clinical studies suggest that these parameters may improve with vitamin D supplementation; however, prospective, randomized and placebo-controlled studies are required to establish firm conclusions.

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MINIREVIEWS

Recombinase polymerase amplification as a promising tool in hepatitis C virus diagnosis

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Key words: Hepatitis C virus; Nucleic acid testing; Polymerase chain reaction; Point-of-care; Recombinase polymerase amplification

Core tip: Recombinase polymerase amplification (RPA) shows many advantages over both real time polymerase chain reaction and other isothermal Amplification methods. In this review we show the importance of molecular detection methods and how isothermal amplification techniques offer molecular point-of-care diagnosis. RPA shows unique characteristics among isothermal approaches that makes it a promising tool in the molecular diagnosis. Because hepatitis C virus is an endemic viral infection, we suggest that RPA may play an important role and save much time in screening infected individuals and managing the therapeutic course.

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Abstract

Hepatitis C virus (HCV) infection represents a significant health problem and represents a heavy load on some countries like Egypt in which about 20% of the total population are infected. Initial infection is usually asymptomatic and result in chronic hepatitis that give rise to complications including cirrhosis and hepatocellular carcinoma. The management of HCV infection should not only be focus on therapy, but also to screen carrier individuals in order to prevent transmission. In the present, molecular detection and quantification of HCV genome by real time polymerase chain reaction (PCR) represent the gold standard in HCV diagnosis and plays a crucial role in the management of therapeutic regimens. However, real time PCR is a complicated approach and of limited distribution. On the other hand, isothermal DNA amplification techniques have been developed and offer molecular diagnosis of infectious dieses at point-of-care. In this review we discuss recombinase polymerase amplification technique and illustrate its diagnostic value over both PCR and other isothermal amplification techniques.

HEPATITIS C VIRUS

Hepatitis C virus (HCV) is a positive-sense single-stranded RNA virus that was first cloned in 1989 and classified as a member of the family Flaviviridae[1]. This viral infection is characterized by high replication rate. It is estimated that about 10^{12} virions per day are produced in a given individual[2]. In addition, its genome exhibits a high degree of sequence variation caused by its error prone RNA polymerase. However, there are 6 characterized genotypes of HCV, 52 subtypes within these genotypes[3]. Humans are the only reservoir for HCV infection; which often leads to an asymptomatic chronic state in 80% of cases with subsequent development to acute liver disease.

An estimated 2%-3% of the world's population is living with HCV infection and each year more than 350000

die of HCV-related complications, including cirrhosis, liver failure or hepatocellular carcinoma[4].

Although hepatitis C is considered to be endemic disease worldwide, there is a high degree of geographical variation in its distribution[5-9]. The prevalence of HCV infection is low, in most European countries where it represents 0.5%-2% of the general population[10,11], Americas, Australia, and South Africa (0.2% to 0.5%)[10]. Intermediate prevalence is reported in Middle East, India and Brazil[7,10]. Egypt recorded the highest prevalence of HCV in the world with about 20% of the population[7,9].

HCV is a blood prone infection, modes of transmission that have been reported include; transfusion of contaminated blood products, organ transplantation from infected donors, intravenous drug use, sexual transmission, public shaving, acupuncture, and invasive hospital procedures with contaminated equipment[12-16]. In Egypt where the highest prevalence in the world has been recorded, the major route of HCV infection was *via* an antischistosomal treatment program, with more than 35 million injections given over a 20-year in the period (1960-1980)[17].

The current standard treatment for chronic hepatitis C is a combination of pegylated interferon alfa and ribavirin. Sustained Virological Response (SVR) represents the endpoint of the treatment regimen, which indicates undetectable HCV RNA 24 wk post treatment[18].

Due to the lack of a vaccine or some form of post-exposure prophylaxis, the number of infected individuals will continue to increase, and in turn HCV-related morbidity and mortality, in the absence of effective care and treatment programs. The management of hepatitis C infection should not only focus on the treatment, but also prevention of infection to reduce the reservoir of infected individuals who can transmit the virus[19,20].

HCV DIAGNOSIS TECHNIQUES

The current laboratory techniques used for HCV diagnosis include: (1) Serological assays (*e.g.*, the enzyme-linked immunosorbent assay, recombinant immunoblot assay, *etc.*); and (2) Molecular assays: Depends on nucleic acid testing (NAT): qualitative [*e.g.*, reverse transcriptase polymerase chain reaction (RT-PCR), TMA, *etc.*] and quantitative (*e.g.,* real time PCR, *etc.*).

Advantages and limitation of serologic assays

The ease of automation and cost-effectiveness made serologic assays the most practical tool in HCV diagnosis[21]. However, antibody detection exhibits many disadvantages including that; detection is limited during the early stages of infection, poor sensitivity (false negative) in hemodialysis patients, immunocompromised patients[22-25], an abundance of false-positives[26] (because recovered patients may stay anti-HCV positive for years) and variability in accuracy between deferent commercial kits.

NAT

NAT detect and quantify HCV RNA and are now considered the gold standard in the diagnosis of HCV infection. In this approach, HCV RNA is extracted from the sample and reverse transcribed into the complementary DNA, which is then amplified into a large number of detectable copies by the polymerase chain reaction (PCR). Unlike antibody detection that could be positive for years after resolving infection, the presence of HCV RNA indicates active infection and it can be detected in 1-2 wk post-infection[27,28]. NAT offers accurate and sensitive diagnosis of HCV without any additional confirmatory test and can be used to diagnose individuals with acute HCV infection. In addition, NAT play a crucial role in the management of antiviral therapies by monitoring HCV RNA level. It determines the basal viral load and monitors the treatment response[29]. Till now, fully automated real-time PCR is the most promising approach in NAT as it is faster, more sensitive and is not prone to contamination.

ADVANTAGES AND LIMITATION OF NAT

The importance of NAT arises from its ability to detect and quantify HCV RNA and in turn detecting the active infection (in contrast to anti-HCV). In addition, it can determine the level of the virus replication. Furthermore, it plays an important role in the antiviral treatment regimens and determines whether a virological response has been occurred or not[30].

However, molecular techniques for HCV diagnosis have many limitations including that; it is of complex procedures, time consuming and technically demanding as it cannot be carried out except in a highly equipped molecular biology laboratory (high cost analytical instruments).

ADVANCES IN HCV DIAGNOSIS

Every day the world takes a step towards NAT which becomes more practical than it was before. The competition between the commercial products enforces the companies to produce more simple, easy to use and cheap assays. In addition to the growing dependence on NAT, the significant advances in HCV diagnosis include using point-of-care (POC) alternatives instead of the routine venous puncture. POC can use specimen matrices such as oral fluid or finger-stick blood. Most existing POC are immunoassays and are now widely used for different applications. POC represent an ideal approach for the management of hepatitis C infection as it can reaches remote areas where the high equipped molecular biology laboratories are limited and in turn shorten the time of results which extend HCV screening. For instance, the development of a molecular point-of-care assay would represent a significant improvement in the field of HCV diagnosis.

ISOTHERMAL DNA AMPLIFICATION

Molecular analytical techniques gain a growing interest. According to the mentioned limitation combining conventional molecular methods, especially real time PCR,

Table 1 Characters of some isothermal amplification techniques[51]

	NASBA	LAMP	SDA	RCA	HDA	RPA
Template	DNA, RNA	DNA[1]	DNA[1]	DNA[1]	DNA[1]	DNA[1]
No. of primers	2	4-6	4	1	2	2
No. of enzymes	3	1	2	2	2	2
Temperature (℃)	41	60-65	37	37	65	30-42
Reaction duration (min)	90-120	60-90	120	60	75-90	20
Denaturation step	Y	N	Y	N	N	N
Inhibition tolerance	N	Y	N	N	Y	Y
Product detection	GE, RT	GE, RT, TE	GE, RT	GE	GE, RT	RT
Multiplex	Y	N	Y	N	Y	Y
Point-of-care	Y	Y	Y	N	Y	Y

[1]RNA can be amplified after the introduction of a reverse transcription step. NASBA: Nucleic acid sequence-based amplification; LAMP: Loop-mediated isothermal amplification; SDA: Strand-displacement amplification; RCA: Rolling circle amplification; HDA: Helicase-dependent amplification; RPA: Recombinase polymerase amplification; GE: Gel Electrophoreses; RT: Real Time; TE: Turbidity; Y: Yes; N: No.

Table 2 Advantages/disadvantages of some isothermal amplification methods

Technique	Advantages	Disadvantages
NASBA	Specifically designed to detect RNA and in turn RNA viruses	Denaturation step
	Power saving (41 ℃)	Less efficient in Amplifying RNA targets out of the range 120-250 bp
LAMP	Highly specific (utilizes 4-6 primers spanning 6-8 distinct sequences)	Primer design is complex
	Tolerance to biological substances	Unable to perform multiplex amplification
	Could be detected by a cheap turbidity-meter	
SDA	Power saving (37 ℃)	Sample prep. needed
		Nuclease selection is complex
		Inefficient in long target sequences
RCA	Power saving (37 ℃)	Primer is complex
	Specific enough to allow SNP analysis	RNA amplification is complex
		Works only with a circular nucleic acid template
HDA	Simple primer design	Expensive enzymes
	Robust to biological substances	
	No initial heating step	
RPA	Power saving (37 ℃)	
	Simple primer design	
	Extremely quick (20 min)	
	No initial heating step	
	Robust to biological substances	

NASBA: Nucleic acid sequence-based amplification; LAMP: Loop-mediated isothermal amplification; SDA: Strand-displacement amplification; RCA: Rolling circle amplification; HDA: Helicase-dependent amplification; RPA: Recombinase polymerase amplification.

there was a demand to develop a simple, sensitive and cost effective technology.

Isothermal DNA amplification is an alternative to PCR-based technique and developed for point-of-care diagnosis[31,32]. In isothermal techniques, amplification reactions are performed at a constant temperature and hence there is no need for expensive thermal cycling instrument. Major practiced isothermal amplification techniques include; nucleic acid sequence-based amplification, loop-mediated isothermal amplification (LAMP)[33], strand-displacement amplification (SDA)[34], rolling circle amplification (RCA)[35], helicase-dependent amplification (HDA)[36] and recombinase polymerase amplification (RPA)[37]. Isothermal DNA amplification techniques are simple, rapid and cost effective with equivalent specificity and sensitivity to PCR, enabling point-of-care diagnostics without the need to high costing equipment[31,32]. However, isothermal amplification approaches differed from each other in terms of operating temperature, reaction duration, mechanism, strengths and weaknesses. Table 1 summarizes the characters of the major practiced isothermal amplification methods.

As a competition between isothermal amplification techniques to perform molecular diagnosis at point-of-care, RCA will be kicked out of the race because it is incompatible with point-of-care diagnosis. Complex primer designing and the inability to perform multiplex amplification eliminates LAMP. The need to a denaturation step and the inability to tolerate inhibitory biological components exit both NABSA and SDA. Finally, RPA beats HAD in being faster and cheaper. Table 2 shows advantages and disadvantages of the major practiced isothermal amplification techniques.

RPA

RPA is an isothermal DNA amplification and detection method[37]. The amplification depends on a specific

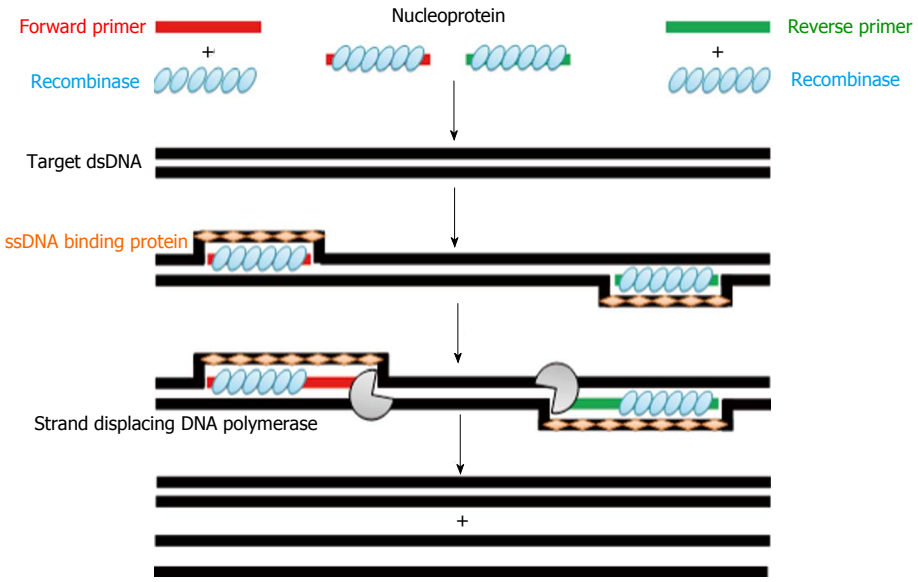

Figure 1 Recombinase polymerase amplification technology amplification cycle (for details, see the text above). dsDNA: Double-stranded DNA.

combination of enzymes and proteins (recombinase, single strand binding protein, and strand displacing DNA polymerase) used at a constant temperature and yielding a result in maximum 10 min. At first, RecA coat a single-stranded DNA (primers) to form nucleoprotein filaments. These filaments can then scan targeted double-stranded DNA for sequences complementary to those of coated primers. Then, the nucleoprotein filaments initiate a 5'-strand invasion at the site of homology (Figure 1) forming what is known as D-loop. The strand invasion is stabilized by single strand binding protein. After that, strand extension takes place at the free 3'-end of the nucleoprotein filaments by a strand displacing DNA polymerase to synthesize a new complementary strand. During strand extension, the new synthesized strand displaces the originally paired strand.

Real-time detection of RPA amplicons is possible *via* specific probes (Figure 2). Development of fluorescence depends on the separation of fluorophore and quencher *via* Exonuclease Ⅲ cleaving at an internal abasic site mimic [tetrahydrofuran (THF)] of the hybridized exo-probe (Figure 2)[38,39]. Fluorescence signal can be measured in real-time *via* a simple point-of-care scanner.

RPA technique is not restricted for amplification of the double stranded DNA targets, but also it could be used for amplification of RNA targets, as in the case with RT-PCR. Ahmed Abd El Wahed *et al*[40], 2013 had developed reverse transcriptase RPA (RT-RPA) assay for the detection of corona virus. The assay showed rapid kinetics with equal sensitivity and specificity of the real-time RT-PCR. The author suggested the diagnostic importance of the RT-RPA assay during the Hajj for the point-of-care detection of MERS-CoV infected cases to prevent the spread of the virus. Euler *et al*[39], 2012 have developed a qualitative real-time RPA assay for detection of *Francisella tularensis* and the assay showed results comparable to real-time PCR. In another wider study by Euler *et al*[41], 2013 RPA based assays were developed for the detection of Gram-negative (*Francisella tularensis* and *Yersinia pestis*) and Gram-positive bacteria (*Bacillus anthracis*), DNA viruses (variola virus), whereas RT-RPA assays were developed for RNA viruses including Rift Valley fever virus, Ebola virus, Sudan virus and Marburg virus. The authors found analytical sensitivity and specificity equal to PCR with no cross-detection among respective targets. Also, Ahmed *et al*[42], 2014 have developed RPA based assay for the detection of *Leptospira* and the method showed fast and less sensitivity to amplification inhibitors. Another competitive character compared to PCR based protocols had been reported by Kersting *et al*[43], 2014 study in which RPA have been used for multiplex detection (detection multiple targets in the same reaction) of *Neisseria gonorrhoeae*, *Salmonella enterica* and *Staphylococcus aureus*. The author concluded that the kinetic performance of RPA was faster than PCR with no loss in sensitivity and specificity[43]. In the light of the above mentioned results it is clear that, RPA show competitive results as compared with PCR as regard to sensitivity and specificity, whereas RPA exceeds PCR as regard to the reaction kinetics.

In another study, RPA showed an impressive results in which the amplification reaction was conducted under a broad range of conditions from 30 ℃-45 ℃ with high inhibitory concentration of known PCR inhibitors in just 15 min[44].

ADVANTAGES AND DISADVANTAGES OF RPA

RPA overcomes the technical difficulties posed by current molecular techniques.

At first; it demonstrates a rapid kinetics, the process begins operating the immediately when the sample is contacted to the reagents and there is no need for melting the double-stranded DNA target.

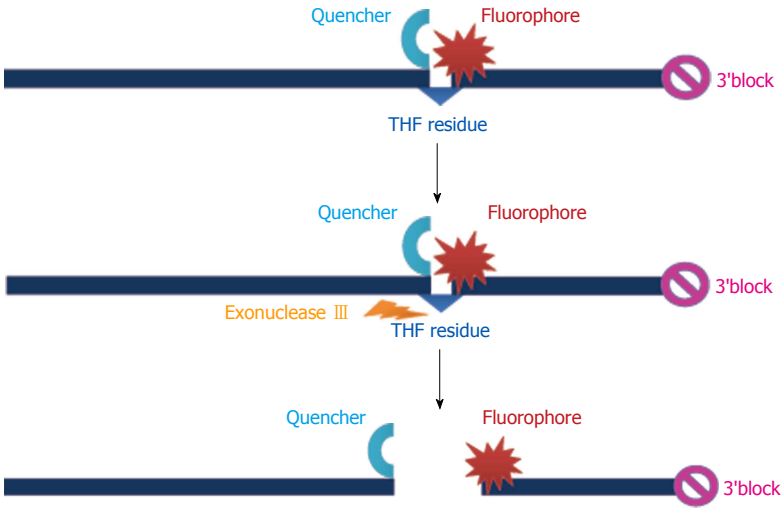

Figure 2 Example for the specific probe of the recombinase polymerase amplification assay. THF: Tetrahydrofuran.

Second, it operates at a constant temperature (30-42 ℃, optimum nearly 37 ℃), which give the advantage of being an energy saving technique and cost saving (there is no need for thermal cycler).

In addition, the target can be either DNA, or RNA, making RPA suitable for the detection and diagnosis of RNA viruses, like HCV.

Furthermore, the combination of probe-based detection, RPA represents a significant advance in the development of portable and accessible nucleic acid-based tests.

Unlike PCR in which the amplification reaction is controlled by temperature, digital RPA suffers from undesired reactions because the amplification could proceeds at room temperature if the nucleic acid sample is premixed with initiation reagents prior to compartmentalization and thus increasing the target count. However, any low-temperature non-specific pre-amplification reaction can be eliminated by compartmentalization of the nucleic acid template prior to adding initiation reagents[45,46].

Another drawback of low temperature amplification results from the interaction between primers even when well-designed. These interactions can create noise that defeats the analysis. However, this drawback could be avoided by using Self Avoiding Molecular Recognition System (SAMRS)[47]. SAMRS are nucleotide analogues that can bind to natural DNA but not to other SAMRS species. Therefore, primers built from SAMRS not interfere with each other. The concept of SAMRS was introduced over a decade ago[48,49] and was exploited to fix interactions between primers in PCR and multiplex PCR[50].

CONCLUSION

Early diagnosis and treatment of HCV infection can reduce the risk of long-term complications and prevent further transmission as well. NAT represent the gold standard for the diagnosis of HCV infection. Detection of HCV RNA level is an important factor in antiviral regimens especially for determination of SVR. RPA combines the advantages of serologic and Molecular techniques and overcome limitations of both. It represents a simple, accurate and cost effective diagnostic tool and can be carried out at remote areas. In turn, it could improve the management of HCV infection by screening carrier individuals and stop transmission. The demand for the development of nucleic acid based point-of-care assay is increasing alongside with increasing the number of HCV infected patients. RPA based HCV diagnosis would represent a significant advance in the management of HCV.

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MINIREVIEWS

Palliative external-beam radiotherapy for bone metastases from hepatocellular carcinoma

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Abstract

The incidence of bone metastases (BMs) from hepatocellular carcinoma (HCC) is relatively low compared to those of other cancers, but it has increased recently, especially in Asian countries. Typically, BMs from HCC appear radiologically as osteolytic, destructive, and expansive components with large, bulky soft-tissue masses. These soft-tissue masses are unique to bone metastases from HCC and often replace the normal bone matrix and exhibit expansive growth. They often compress the peripheral nerves, spinal cord, or cranial nerves, causing not only bone pain but also neuropathic pain and neurological symptoms. In patients with spinal BMs, the consequent metastatic spinal cord compression (MSCC) causes paralysis. Skull base metastases (SBMs) with cranial nerve involvement can cause neurological symptoms. Therefore, patients with bony lesions often suffer from pain or neurological symptoms that have a severe, adverse effect on the quality of life. External-beam radiotherapy (EBRT) can effectively relieve bone pain and neurological symptoms caused by BMs. However, EBRT is not yet widely used for the palliative management of BMs from HCC because of the limited number of relevant studies. Furthermore, the optimal dosing schedule remains unclear, despite clinical evidence to support single-fraction radiation schedules for primary cancers. In this review, we outline data describing palliative EBRT for BMs from HCC in the context of (1) bone pain; (2) MSCC; and (3) SBMs.

Key words: Hepatocellular carcinoma; Metastasis; Radiotherapy; Palliative therapy; Spinal cord compression; Skull base metastasis

Core tip: Due to a lack of clinical data, external-beam radiotherapy (EBRT) for bone metastases (BMs) from hepatocellular carcinoma (HCC) is still not widely used as a palliative therapy component, and the optimal dosing schedule remains unclear. BMs from HCC typically occur as expansive, bulky soft-tissue masses; they exhibit expansive growth that compresses the peripheral nerves, spinal cord, or cranial nerves, causing both bone and neuropathic pain, and neurological symptoms. In this review, we outline the data describing palliative EBRT for BMs from HCC to treat bone pain, spinal compression, and skull base metastases.

Hayashi S, Tanaka H, Hoshi H. Palliative external-beam radiotherapy for bone metastases from hepatocellular carcinoma. *World J Hepatol* 2014; 6(12): 923-929 Available from: URL: http://www.wjgnet.com/1948-5182/full/v6/i12/923.htm DOI: http://dx.doi.org/10.4254/wjh.v6.i12.923

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer in men worldwide[1]. The rate of bone metastases (BMs) in extrahepatic metastasis is reported to be approximately 20%[2]. The incidence of BMs in HCC patients has historically been low compared with those of other cancers, but has recently increased[3]. Previously,

Table 1 Incidence of bone metastases in clinical studies *n* (%)

Ref.	Study period	Patients	Extrahepatic Ms	Incidence of BMs	Rate of BMs in extrahepatic Ms
Kuhlman *et al*[13]	1979-1985	300		22 (7.3)	
Liaw *et al*[14]	1983-1887	395		20 (5)	
Katyal *et al*[15]	1992-1997	403	148 (36.7)	41(10.2)	28
Fukutomi *et al*[3]	1978-1987	269		12 (4.5)	
	1988-1997	404		52 (12.9)	
Natsuizaka *et al*[5]	1995-2001	482	65 (13.5)	25 (5.2)	38.50
Uchino *et al*[2]	1990-2006	2386	342 (14.3)	87 (3.6)	25.40
Senthilnathan *et al*[16]	2000-2008	209	51 (18)	5 (2)	10

BMs: Bone metastases; Ms: Metastases.

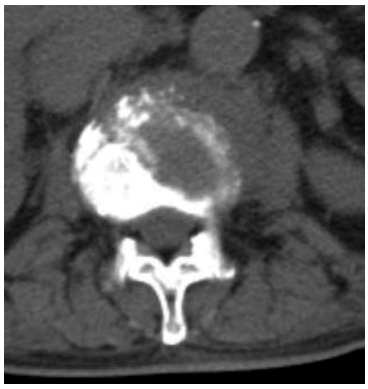

Figure 1 Typical computerized tomography image of a lumbar spinal bone metastasis from hepatocellular carcinoma. The bone metastasis shows osteolytic, destructive, and expansive components with soft-tissue masses.

clinicians did not focus on BMs in advanced HCC because of their low incidence, and the prognosis of these patients was generally poor[4]. Recently, the prognosis and management of HCC have improved as a result of novel imaging techniques and multidisciplinary treatment approaches. BMs are now diagnosed more frequently in HCC patients with extrahepatic metastases[5]. BMs themselves rarely affect patient survival; however, they are the most common source of moderate and severe cancer pain[6,7] and can cause neurological symptoms[7]. In patients with spinal BMs, the consequent metastatic spinal cord compression (MSCC) causes paralysis. Skull base metastases (SBMs) with cranial nerve involvement can cause neurological symptoms. External-beam radiotherapy (EBRT) can effectively relieve bone pain[7,8] and the neurological symptoms caused by BMs[7,9]. However, EBRT has not been widely used in the palliative management of BMs from HCC because only a few reports have been published that focus on its use to relieve pain and neurological symptoms. In this review, we will outline the data pertaining to the use of EBRT for BMs from HCC.

INCIDENCE OF BMs

HCC is accompanied by BMs in 6%-20% of patients in autopsy studies[10-12]. The incidence of BMs in HCC patients has been reported to be relatively low; 2%-12.9% in clinical studies, and 7.3%-38.5% in patients with extrahepatic metastases (Table 1)[2,3,5,13-16]. However, BM incidences of 10.2% and 12.9% were reported by Katyal *et al*[15] and Fukutomi *et al*[3], respectively, which are higher than previously reported rates. According to Katyal *et al*[15], this difference may have been the result of current treatment regimens that utilize combined chemotherapy and chemoembolization to prolong survival. Fukutomi *et al*[3] compared the incidence of BMs in two chronological periods (1987-1997 and 1998-1997). BMs were found in 4.5% of patients in the first decade and 12.9% of patients in the second decade. The increased incidence of bone metastasis was attributed to the prolonged survival of HCC patients due to improved diagnosis and treatment. Bone scintigraphy and computed tomography (CT) have been used widely as imaging modalities for BMs, and magnetic resonance imaging (MRI) has often been performed in recent studies. MRI is useful in cases in which the bone scan is negative and the BMs have properties of soft tissue masses[17]. In three recent studies, the incidence of BMs was reported to be 2%-5.4%[2,5,16]. However, the rate of BMs in patients with extrahepatic metastases is relatively high (25.4%-38.5%)[2,5], except in one report from the United States[16]. In a recent study, Natsuizaka *et al*[5] found that extrahepatic metastases were relatively common, and more than 65% of the study patients had early-stage tumors that would not be expected to metastasize.

RADIOLOGICAL FEATURES

Most BMs from HCC are located in the vertebrae, followed by the pelvis, ribs, sternum, limb bones, and cranium[18], which is a similar distribution to the BMs of other tumor types[19]. The distribution of BMs in HCC is similar to that observed in previous studies[3,4,8]. The lower-thoracic and lumbar vertebrae are common sites for vertebral BMs[18]. The reported incidence of skull metastases varies widely; 3.5%-30%[4,7,8,14].

Radiographically, typical BMs from HCC appear as expansible, destructive findings with large soft-tissue masses[13]. Most BMs are osteolytic and thus detectable using CT[14,19] (Figure 1). However, HCC patients rarely exhibit either purely osteolytic or osteoblastic lesions[13,19]. Soft-tissue masses are unique to BMs of HCC and have been observed in 39%-85.4% of patients[8,18-20]. These

Table 2 Studies of external radiotherapy to treat painful bone metastases from hepatocellular carcinoma

Ref.	Patients (*n*)	Sites (*n*)	Fraction dose	Pain relief (CR)	Dose-response relationship	Comments
Roca *et al*[27]	26	37	MFs 30-50 Gy	79% (44%)	NR	11 lesions (with CTx)
Kaizu *et al*[28]	57	99	MFs 20-65 Gy	83.8% (33%)	Better pain relief TDF ⩾ 77	16 lesions (with TAE) Tumor volume (NS)
Matsuura *et al*[29]	38	44	MFs 26-60 Gy	91% (32%)	NR	Tumor regression (> 40 Gy)
Seong *et al*[7]	51	77	MFs 12.5-50 Gy	73%	Better pain relief BED > 43 Gy	With neurological symptoms (25%)
He *et al*[8]	205	205	MFs 32-66 Gy	99.5% (29.80%)	NR	Higher retreatment rate (with soft-tissue masses)
Hayashi *et al*[30]	28	48	MFs 20-52 Gy SF 8 Gy	83% (17%) MFs: 87% (17%) SF: 75% (17%)	NR	Longer pain relief (> 36 Gy) No spinal compression No neuropathic pain

MFs: Multiple fractions; SF: Single fraction; CR: Complete relief; NR: No dose response; TDF: Time, dose, and fractionation factor; BED: Biologically effective dose; NS: No significant difference; CTx: Chemotherapy; TAE: Transcatheter arterial embolization.

soft-tissue masses can replace the normal bone matrix and exhibit expansive growth, frequently within the vertebral body. Paravertebral masses have been shown to grow inward to encapsulate and destroy the bone matrix[18]. These masses often compress peripheral nerves, the spinal cord[21], or cranial nerves[22], causing not only bone pain but also neuropathic pain[6] and neurological symptoms.

RADIOTHERAPY FOR BONE PAIN

EBRT is prescribed most frequently to relive pain from BMs and the efficacy of EBRT for treating BMs has been well established[23]. Generally, pain relief is obtained in 60%-90% of treated patients, but the sites of primary tumors from these reports were mainly in the lung, breast, and prostate[23-26]. There have only been a few studies of EBRT for HCC BMs, but they show that 73%-99.5% of patients obtained overall pain improvement and 17%-44% of patients achieved complete pain relief (Table 2)[7,8,27-30]. Except for a report from China[8], these studies were retrospective analyses with a small sample size; however, the reported results for overall pain relief are similar to those obtained for BMs of other primary tumors. HCC is often complicated by liver failure, and narcotic drugs may induce hepatic coma. Therefore, EBRT may play an important role in relieving the pain from BMs, thus minimizing the use of narcotic drugs for pain relief.

Soft-tissue masses with BMs often cause neuropathic pain, spinal compression, and pathological fractures, and these issues were evaluated simultaneously with bone pain relief in some previous reports. The pain assessments differed among the studies; two recent studies[8,30] evaluated pain relief using the International Bone Metastases Consensus Working Party Guidelines[31,32] with some modifications. Even after considering the differences among studies, it has been shown that EBRT is equally effective for the relief of pain caused by BMs from HCC, as well as metastases from other primary tumors.

Various EBRT dosage and fractionation schedules have been used to treat pain, ranging from an 8 Gy single fraction (SF) to multiple fractions (MFs). An SF of 8 Gy delivered in one day is more convenient for the patient and more cost effective compared with schedules employing MFs. However, MFs that deliver a higher total dose than an SF may have increased biological effects on the tumor. In a randomized controlled trial conducted by the Radiation Therapy Oncology Group (RTOG 9701)[33] in which an SF (8 Gy) was compared with MFs (30 Gy in 10 fractions over 2 wk), it was demonstrated that both schedules provided equivalent pain relief. Furthermore, the RTOG trial found a significantly lower rate of acute toxicity with an SF compared to MFs, although there was no significant difference in late toxicity (*e.g.*, pathologic fractures). Similar findings concerning the pain relief after treatment based on an SF or MFs have also been reported[26,34]. Similarly, according to other recent studies including a meta-analysis, both SF and MF-based treatments have provided equivalent pain relief, although SF treatment often requires re-treatment[23-25,35]. In terms of pain relief, most previous studies failed to show a dose-response relationship for BMs from other primary cancers. For BMs from HCC, Roca *et al*[27], Matsuura *et al*[29], and He *et al*[8] also found no apparent dose-response relationship for pain relief. In contrast, Kaizu *et al*[28] and Seong *et al*[7] did find a dose-response relationship, although in the former study, 15 of the 57 patients analyzed[28] underwent transcatheter arterial embolization of BMs in addition to EBRT, and 25% of the patients in the study by Seong *et al*[7] had neurological symptoms with bone pain. He *et al*[8] also found no dose-response relationship for pain relief, but higher complete pain relief rates were obtained using higher radiation doses. Furthermore, they observed that the re-treatment rate was higher among patients with expansible soft-tissue masses and noted an increased presence of residual cancer cells in these patients relative to those lacking soft-tissue extension. Matsuura *et al*[29] reported a lack of observed tumor regression at doses < 40 Gy and that 3 patients treated

with doses ≥ 40 Gy (40 Gy, 46 Gy, and 60 Gy) survived for > 6 years without recurrence. Pain caused by BMs can originate directly from the bone, or as a result of nerve root compression, or muscle spasms in the lesion area (*i.e.*, neuropathic pain)[7]. In a randomized trial of radiotherapy for neuropathic pain caused by BM, Roos *et al*[36] (Trans-Tasman Radiation Oncology Group) compared the efficacy of SF (8 Gy) to MFs (20 Gy/5 fraction) treatment and concluded that an SF was not as effective as MFs; the outcomes with SF treatment were generally poor, although the difference was not statistically significant. In that study, the most frequent primary tumor sites were the lung and prostate. For patients with BMs from HCC that cause neuropathic pain through nerve root compression, a higher radiation dose may be needed to shrink the soft-tissue mass and provide pain relief.

Nearly all previous studies[7,8,27-29] involving BMs of HCC used MF schedules and evaluated both bone pain and neuropathic pain. We conducted a retrospective evaluation of the palliative efficacy of EBRT, excluding cases with spinal cord compression or neuropathic pain[30] and assessed different dosing schedules for BMs from HCC with soft-tissue masses. Our analysis included a relatively small number of patients (28 patients, 42 sites), and the overall response rates were 75% and 87% for SF and MF treatment, respectively; this difference was not significant. Patients undergoing high-dose MFs (≥ 36 Gy in total) achieved on average a significantly longer duration of pain relief than those undergoing SF or moderate-dose MF therapy (≤ 30 Gy in total). The median durations of overall pain relief for MFs were 3.8 and 1.8 mo after SF treatment. These results were similar to those reported for other series involving different primary cancers[24,25,34]. In our study, we found that EBRT effectively palliated painful BMs from HCC, that an 8 Gy SF and MFs resulted in equivalent pain relief, and that high-dose MF schedules may result in longer lasting pain relief.

Soft-tissue masses are unique to BMs from HCC and often cause both bone and neuropathic pain. In HCC patients with neuropathic pain, higher RT doses using an MF schedule are usually necessary because of the presence of soft-tissue masses. It is critical to discriminate between these different pain types, and large-scaled cohort studies are necessary to determine an optimal radiotherapy plan in terms of doses and fractions for each.

RADIOTHERAPY FOR SPINAL BMs

Spinal BMs often cause not only pain but also MSCC, which primarily develops in one of three ways[37]: (1) the continued growth and expansion of vertebral BMs into the epidural space; (2) neural foraminal extension *via* a paraspinal mass; and (3) the destruction of vertebral cortical bone, leading to vertebral body collapse and the displacement of bony fragments into the epidural space. SCC secondary to BMs from HCC can develop in any of these ways because typical these metastases have an expansible and destructive nature and give rise to soft-tissue masses[13].

MSCC is estimated to occur in approximately 5%-10% of all cancer patients[37]. The most common primary sites are the breast and lung[37,38]; however, the rate of MSCC resulting from BMs from HCC is unclear. MSCC that diminishes motor function and causes paraplegia is considered an oncological emergency requiring urgent treatment[38]. Conventionally, MSCC has been managed with corticosteroids and high-dose EBRT. EBRT is an effective treatment for MSCC and has been included in standard care. Rades *et al*[38] retrospectively analyzed the use of 5 radiotherapy schedules (8 Gy, 20 Gy/5 fractions, 30 Gy/10 fractions, 37.5 Gy/15 fractions, and 40 Gy/20 fractions) for MSCC treatment and found that motor function improved by 26%-31%, post-treatment ambulation was achieved in 63%-74% of cases, and that all 5 schedules provided similar functional outcomes. Rades *et al*[38] therefore recommended a schedule of 8 Gy for patients with a poor survival prognosis and 30 Gy/10 fractions for other patients. Maranzano *et al*[39] reported that 76% of patients achieved full recovery, or at least were still able to walk, after EBRT with doses > 30 Gy over a 2 wk period in combination with steroids, and that the most important response predictors were an early diagnosis and favorable histology. For MSCC specifically caused by HCC, Maranzano *et al*[39] reported a median duration of improvement of only 1 mo, which was shorter than the duration observed for other cancers, including breast cancer, for which it was 12 mo. Nakamura *et al*[9] reported a retrospective series of 24 ambulatory patients with MSCC derived from HCC. Five patients (21%) underwent salvage therapy and 4 (21%) had become non-ambulatory by the last follow-up. The ambulatory rates at 3 and 6 mo were 85% and 63%, respectively. Nakamura *et al*[9] concluded that EBRT with a biologically effective dose range of 39-50.7 Gy (total radiation dose range, 30-39 Gy) was not sufficient to prevent MSCC-related paralysis and that dose escalation *via* a precise radiation technique should be evaluated. In MSCC caused by BMs from HCC, it will probably be important to shrink and control the soft-tissue masses. Therefore, higher radiation doses are needed to prevent MSCC-related paralysis. For patients with a good survival prognosis, high-precision radiation therapy [intensity-modulated radiotherapy (IMRT) or stereotactic irradiation (STI)] should be considered for the delivery of higher radiation doses, whilst sparing the spinal cord and reducing the risk of radiation myelitis. In addition to EBRT, surgery is being re-evaluated for the palliative management for MSCC. The results of a randomized trial reported by Patchell *et al*[40], showed that direct decompressive surgery with postoperative EBRT was more effective at restoring ambulation than EBRT alone.

Vargas *et al*[41], Somerset *et al*[42] and Doval *et al*[21] showed in case reports that patients with MSCC derived from HCC who were treated with laminectomy or resection of the epidural lesion had a good clinical course. Vargas *et al*[41] concluded from their case report that surgi-

cal therapies such as direct decompression of the tumor with postoperative EBRT or vertebral body resection with stabilization should be considered in patients for whom surgery could be expected to succeed.

RADIOTHERAPY FOR SBMs

SBMs occur in 4% of cancer patients[43] and often cause pain or cranial nerve palsies. Because of their rarity, SBMs have received limited attention in the published medical literature. Their clinical manifestation depends on the metastatic cranial nerve invasion site. In a review by Laigle-Donadey *et al*[43], the most common primary cancers from which SBMs originated were prostate (38%) and breast cancer (20%). The incidence of SBM from HCC has been reported to be 0.4%-1.6%[44-47] and until 2009, only 25 such cases had been reported[38]. However, the incidence of SBM from HCC increased significantly during the period between 1990 and 2001[39]. SBM without other osseous metastases is an unusual finding and cranial nerve deficits are found in 96% of cases in which the SBM was derived from HCC[38]. Radiotherapy is usually the standard treatment for SBM and has been used to treat 70% of patients[43]. EBRT provides excellent pain relief and often leads to the regression of cranial nerve dysfunction, which lasts until death in most cases. There is consensus that the rate of neurological improvement is closely related to the length of time to EBRT following the onset of symptoms[43]. Vikram *et al*[48] reported that 87% of patients for whom EBRT was initiated < 1 mo after the onset of symptoms in contrast to 25% for whom EBRT was initiated ⩾ 3 mo after the onset of symptoms. However, the appropriate doses and fractions to use in EBRT for SBMs have still not been agreed upon. Discrepancies with respect to the dose-response relationship can be explained by the different radiosensitivity of each primary tumor. In cases involving SBMs from HCC, higher radiation doses are needed to improve the neurological symptoms by resolving the compression and invasion of cranial nerves caused by soft-tissue masses. Nozaki *et al*[47] reported a case in which multiple SBMs from HCC were successfully treated with EBRT. They found that slightly higher radiation doses (50 Gy/20 or 25 fractions) were delivered and improvements in neurological symptoms and tumor regression were achieved. However, EBRT places certain organs at risk, including the brain stem, optic nerve, and optic chiasm. Stereotactic radiosurgery (SRS) and stereotactic radiotherapy (SRT) are more recent therapeutic options for SBMs. They are high-precision radiation therapies in which delivery is accurate to within one to two millimeters and are performed in a non-surgical procedure that delivers precisely targeted radiation at much higher doses than traditional radiotherapy in a single dose (SRS) or fractionated regimen (SRT). This treatment is only possible because of the development of highly advanced radiation technologies such as the gamma knife, and high-precision linear accelerators that permit maximum dose delivery within the target while minimizing the dose to organs at risk. In a report of HCC cases treated by gamma knife radiosurgery, the clinical symptoms improved in 61% of the patients after treatment and tumor control was achieved in 67% of cases[49]. Gamma knife radiosurgery is particularly useful for small tumors (diameter < 30 mm)[36]. Stereotactic radiotherapy *via* Novalis, a high-precision linear accelerator, can administer high doses to tumors while sparing normal structures and organs at risk, thus being a useful EBRT technique for SBM treatment[50]. In a study utilizing Novalis, all 11 cases (including 1 HCC case) achieved and maintained local control until the end of the follow-up period or death. SBM remains a challenge with respect to EBRT planning and delivery.

SURVIVAL ASSOCIATED WITH BMs

The 1-year survival rate after EBRT initiation or the diagnosis of BMs has been reported to be 13.8%-32.4%, with a 5-7.4 mo median survival time[7,8,13,29,30]. Unfavorable significant prognostic factors of patients with BMs have been reported as lower performance status, multifocal BMs, tumor stage ⅣA, metastasis to other organs, higher tumor marker levels, uncontrolled intrahepatic tumors, and ascites at the initial presentation[4,7,8,29].

The prognosis of patients with MSCC is worse than for patients with only BMs. According to Nakamura *et al*[9], the median observed survival duration for all patients was 5.1 mo and the overall 6-mo survival rate was 38%.

SBMs are generally late events and occur at a stage when many patients have already developed disseminated disease, particularly other BMs[43]; 71% of these patients were reported to have died within a short period of between 11 d and 9 mo after the onset of neurological symptoms[40].

CONCLUSION

Soft-tissue masses are unique to BMs from HCC and often cause both bone and neuropathic pain, and neurological symptoms. EBRT is effective for the relief of painful symptoms resulting from BMs, MSCC, and SBMs from HCC. However, the optimal dose and fraction schedules for bone pain palliation remain unclear. Large-scaled cohort studies are necessary to determine the optimal radiotherapy doses and fractions to treat both bone pain and neuropathic pain. MSCC and SBMs remain a challenge for EBRT. High-precision radiation therapy (IMRT or STI) should be considered for the delivery of higher radiation doses with sparing of normal tissues.

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RETROSPECTIVE STUDY

Evaluation of hepatocellular carcinoma development in patients with chronic hepatitis C by EOB-MRI

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Abstract

AIM: To evaluate the efficacy of ethoxibenzyl-magnetic resonance imaging (EOB-MRI) as a predictor of hepatocellular carcinoma (HCC) development.

METHODS: Between August 2008 and 2009, we studied 142 hepatitis C virus-infected patients (male 70, female 72), excluding those with HCC or a past history, who underwent EOB-MRI in our hospital. The EOB-MRI index [liver-intervertebral disc ratio (LI)] was calculated as: (post-liver intensity/post-intervertebral disc intensity)/(pre-liver intensity/pre-intervertebral disc intensity).

RESULTS: The median follow-up period was 3.1 years and the patients were observed until the end of the study period (31 December, 2012). In the follow-up period, HCC occurred in 21 patients. The cumulative occurrence rates were 2.1%, 9.1%, and 14.1% at 1, 2, and 3 years, respectively. Using the optimal cut-off value of LI 1.46, on univariate analysis, age, aspartate amino transferase (AST), α-fetoprotein (AFP) ⩾ 10, albumin, total cholesterol, prothrombin time, platelets, and LI < 1.46 were identified as independent factors, but on multivariate analysis, LI < 1.46: risk ratio 6.05 (1.34-27.3, P = 0.019) and AFP ⩾ 10: risk ratio 3.1 (1.03-9.35, P = 0.045) were identified as independent risk factors. LI and Fib-4 index have higher area under the receiver operating characteristic curves than other representative fibrosis evaluation methods, such as Forn's index and AST-to-platelet ratio index.

CONCLUSION: LI is associated with the risk of HCC occurrence in hepatitis C patients. LI may be a substitute for liver biopsy when evaluating this risk and its combined use with Fib-4 is a better predictive method of HCC progression.

Key words: Ethoxibenzyl-magnetic resonance imaging; Hepatocellular carcinoma; Risk factor; Fibrosis

Core tip: This manuscript addresses a method of hepatocellular carcinoma (HCC) prediction by using a new technique that evaluates hepatic fibrosis using a non-invasive method (reported recently). This is the first reported study to consider a possible substitute for liver biopsy by using an magnetic resonance imaging (MRI) method (a widespread method in public medical services) for evaluating the risk of occurrence. We propose that this method will become one of the most popular and precise noninvasive methods to predict the occurrence of HCC, and the combination of this MRI method and Fib-4 index may provide a better predictive method of HCC progression.

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INTRODUCTION

The major cause of cirrhosis globally is chronic hepatitis C. The risk of hepatocellular carcinoma (HCC) development is related to this, as reported in several papers[1-3], and advanced fibrosis increases the risk of carcinogenesis[1]. The prognosis of HCC is not good, even when detected and treated at an early stage[4-6]. Thus, it is important to determine outpatients' fibrotic stage in order to identify the risk of HCC occurrence in the management of patients with chronic liver disease. Even now, to determine the grade of fibrosis, the gold standard is liver biopsy, but it is associated with certain problems such as sample error and severe complications[7-10]. Previously, noninvasive methods to evaluate fibrosis were reported, such as Forn's index[11], the Fibro index[12], and aspartate amino transferase-to-platelet ratio index (APRI)[13]. Using laboratory data, it has been reported that the Fibrotest is a useful prognostic factor for hepatitis C patients[14]. On the other hand, specific methods, such as transient elastography[15], magnetic resonance (MR) elastography[16], and acoustic radiation force impulse[17], have been reported to evaluate fibrosis as surrogates of liver biopsy. Transient elastography is reported to indicate a wide-ranging risk of HCC incidence. We recently reported the accuracy of staging fibrosis in chronic hepatitis in hepatitis C virus (HCV) infection using ethoxibenzyl-MR imaging (EOB-MRI)[18], but there are no reports about a predictor of HCC incidence using this new method. Here, we report a study to evaluate the efficacy of EOB-MRI as a predictor of HCC development.

MATERIALS AND METHODS

Patients

Between August 2008 and December 2009, we studied 142 HCV-infected patients, excluding those with HCC or a past history, who underwent EOB-MRI in our hospital. Clinical data were obtained within one month of EOB-MRI information being obtained. The definition of HCV infection was determined by a positive anti-HCV antibody and detection of quantitative or qualitative HCV RNA. Exclusion criteria were as follows: (1) infection with hepatitis B or human immunodeficiency viruses; (2) alcohol abuse; (3) the presence of numerous liver tumors; and (4) having previously undergone partial splenic arterial embolization or splenectomy. During the follow-up period, the history of interferon (IFN) therapies and associated responses was examined. We defined a sustained virological response (SVR) as undetectable HCV-RNA for at least 24 wk after IFN therapy. The study protocol conformed to the ethics guidelines of the 1975 Helsinki Declaration and was approved a priori by the institution's human research committee. All blood tests were performed within 1 wk before or after MRI.

Follow-up of patients and HCC diagnosis

The screening of HCC occurrence was carried out by enhanced MRI or enhanced computed tomography (CT). Outpatients were followed up with blood tests, tumor markers for HCC, and image analysis, such as ultrasonography, enhanced CT, or enhanced MRI, every 3 to 6 mo. The diagnosis of HCC was determined by enhanced CT or enhanced MRI, considering enhancement in the arterial phase and washout in the earlier delayed venous phase as a classical sign of HCC[19,20]. When the diagnosis of HCC was not clear in CT or MRI, a histological diagnosis was performed by tumor biopsy[21]. Cases that were diagnosed as HCC within 6 mo from the first MRI trial were excluded because there should have been only small HCC when the first MRI was performed. This study was continued until December 31, 2012.

MRI techniques

A 1.5-Tesla MR system (Philips Co., Amsterdam, The Netherlands) was used: 0.025 mmol/kg body weight gadoxetate disodium was intravenously injected and quantitative measurements were performed using unenhanced and gadoxetate disodium-enhanced imaging at 20, 35, 70, and 180 s, and the imaging at 15, 20, and 25 min was obtained as hepatobiliary phases. Imaging parameters were as follows: repetition time/echo time = 4.17/2.05 ms. Then, 1-2 cm^2 regions of interest of the mean signal intensity value of the liver were measured. At each MRI, the means of three different regions of right anterior, right posterior, and left lateral segments of the liver devoid of large vessels or severe artifacts were calculated. Using the liver to intervertebral disk signal intensity (LISI) and liver signal intensity/intervertebral disk signal intensity, we calculated the post-enhanced LISI/pre-enhanced LISI [described as liver-intervertebral disc ratio (LI)], as detailed in our previous report[18]. We used hepatobiliary phase data at 20 min because this is most commonly used globally and the data showed no significant difference from the value at 25 min. As we reported previously, because cut-off values of 1.31 and 1.80 are representative values of liver cirrhosis and significant fibrosis of the liver, we divided all patients into < 1.31, 1.311 to 1.38, 1.381 to 1.50, 1.501 to 1.60, and > 1.601. Age, sex, aspartate aminotransferase (AST), alanine aminotransferase (ALT), serum albumin level, total bilirubin (T.Bil), gamma-glutamyl transpeptidase (γGTP), total cholesterol, and platelet count (Plt) were examined. The prothrombin time (PT) was measured as a percentage of the daily internal control.

Statistical analysis

Baseline data are presented as the mean ± SD with the range in parentheses for quantitative variables. The best models derived from the categorical variables were compared by the χ^2 or Fisher's exact test, whereas Wilcoxon rank sum test (nonparametric) for continuous variables and the unpaired Student's *t* test (parametric) were used to evaluate differences in age, sex, albumin, T.Bil, PT, Plt, AST, ALT, γGTP, total cholesterol, and α-fetoprotein (AFP) at the time of entry. The results are reported as

Table 1 Baseline characteristics of 142 patients with chronic hepatitis C

Variables	Mean ± SD
Age (yr)	66.1 ± 12.4 (28-87)
Male (M/F)	70/72
AST (U/L)	48.9 ± 23.4 (11-155)
ALT (U/L)	51.7 ± 34.1 (10-228)
Serum albumin (g/dL)	4.1 ± 0.5 (2.4-5)
Gamma-GT(IU/L)	67 ± 92 (14-811)
ALP (U/L)	331 ± 171 (141-1206)
T.Chol (mg/dL)	175 ± 36 (90-280)
T.Bil (mg/dL)	0.86 ± 0.42 (0.3-2.9)
PT (%)	93 ± 15.2 (55.2-134)
Platelet ($\times 10^3/\mu L$)	136 ± 60 (42-338)
AFP (ng/mL)	14.5 ± 27.5 (1.6-235)
LI	1.51 ± 0.19 (1.11-2.15)
Patients who received IFN, *n* (%)	39 (27.5)
Patients who achieved SVR, *n* (%)	27 (19.0)

AST: Aspartate amino transferase; ALT: Alanine aminotransferase; Gamma-GT: Gamma-glutamyl transpeptidase; T.Chol: Total cholesterol; T.Bil: Total bilirubin; PT: Prothrombin time; AFP: α-fetoprotein; LI: Liver-intervertebral disc ratio; IFN: Interferon; SVR: Sustained virologic response; ALP: Alkaline phosphatase; F: Female; M: Male.

hazard ratios with 95%CI. $P < 0.05$ in a two-tailed test was considered significant for all analyses. Patients were censored when they died without HCC development, when they stopped visiting, or when the study period ended. Cumulative occurrence curves were analyzed using the Kaplan-Meier method and tested by Wilcoxon's method. The Cox proportional hazard regression model was used to estimate the risk factors for hepatocarcinogenesis using the following variables in univariate and multivariate analyses: sex, albumin, T.Bil, PT, Plt, AST, ALT, γGTP, alkaline phosphatase (ALP), total cholesterol, AFP (≥ 10 ng/mL), LI (< 1.46) at the time of entry, and the history of IFN therapy (with or without, and SVR or non-SVR).

All statistical analyses were performed using IBM SPSS Statistics 21 software (IBM, Chicago, IL, United States).

RESULTS

Patient characteristics

A total of 145 patients who had undergone EOB-MRI were examined. Three patients were excluded because they developed HCC within 6 mo.

Patient characteristics at the time of EOB-MRI are shown in Table 1. There were 70 men and 72 women, with a mean age of 66.1 ± 12.4 years. The mean AFP level was 14.5 ng/mL and the median was 5 ng/mL. Thirty-seven patients (26%) had an AFP level of ≥ 10. Thirty-nine patients received IFN and 27 patients achieved SVR in the follow-up period.

Occurrence of HCC and patient follow-up

The median follow-up period was 3.1 years, during which 14 (9.8%) patients were lost to follow-up and were censored at the time of the last visit. Nine patients died of liver failure, one died of gastroenterological varices rupture, and nine died of liver-unrelated causes, and they were censored when they died. The remaining patients were observed until the end of the study period (31 December, 2012). During the follow-up period, HCC occurred in 21 patients. The cumulative HCC occurrence rates were 2.1%, 9.1%, and 14.1% at 1, 2, and 3 years, respectively, by the Kaplan-Meier method. Baseline characteristics were compared in patients with and without HCC occurrence (Table 2). There were no significant differences between the no-HCC occurrence group and the HCC occurrence group in terms of age, sex, ALT level, gamma-GT, T.Bil, the performance of IFN therapy, and the achievement of SVR, while AST, ALP, and AFP were higher and albumin, total cholesterol (T.Chol), PT, platelets, and LI were lower in the HCC occurrence group than in the no-HCC occurrence group.

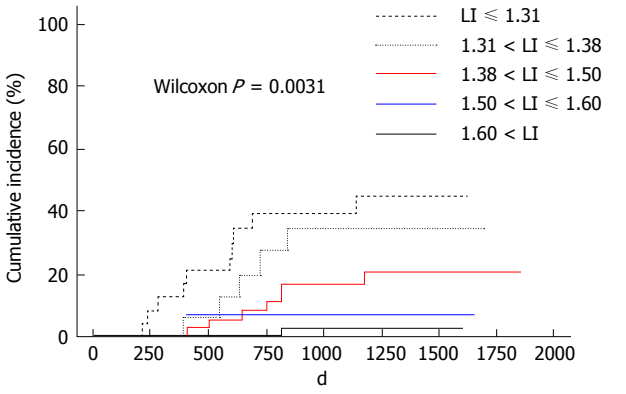

Figure 1 Cumulative incidence of hepatocellular carcinoma occurrence stratified by liver-intervertebral disc ratio. Cumulative occurrence rates increased gradually in an LI-independent manner. LI: Liver-intervertebral disc ratio.

Occurrence rate of HCC stratified by LI

The cumulative occurrence rates at 1, 2, and 3 years in each LI group were 0%, 0%, and 2% in patients with LI ≥ 1.601; 0%, 5.8%, and 5.8% in patients with LI 1.501-1.600; 0%, 7.1%, and 14.3% in patients with LI 1.381-1.500; 0%, 11.8%, and 23.5% in patients with LI 1.311-1.380; and 12.5%, 29.2%, and 33.3% in patients with LI ≤ 1.310, respectively (Figure 1). The occurrence rates differed significantly among the 5 LI groups ($P = 0.0031$), increasing with decreasing LI.

The receiver operating characteristic curve (ROC) curve was used to evaluate the cumulative incidence of LI and a cut-off value of 1.46 was determined [area under the ROC (AUROC): 0.765 ± 0.05, 0.669-0.861] by calculating the highest accuracy value (0.63) and likelihood ratio (2.19). The use of this cut-off value resulted in sensitivity: 90.5%, specificity: 58.7%, positive predictive value: 27.5%, and negative predictive value: 97.3%. We compared these results with several representative fibrosis evaluation methods reported previously (Figure 2). The AUROC for each was: Forn's index, 0.733 ± 0.05,

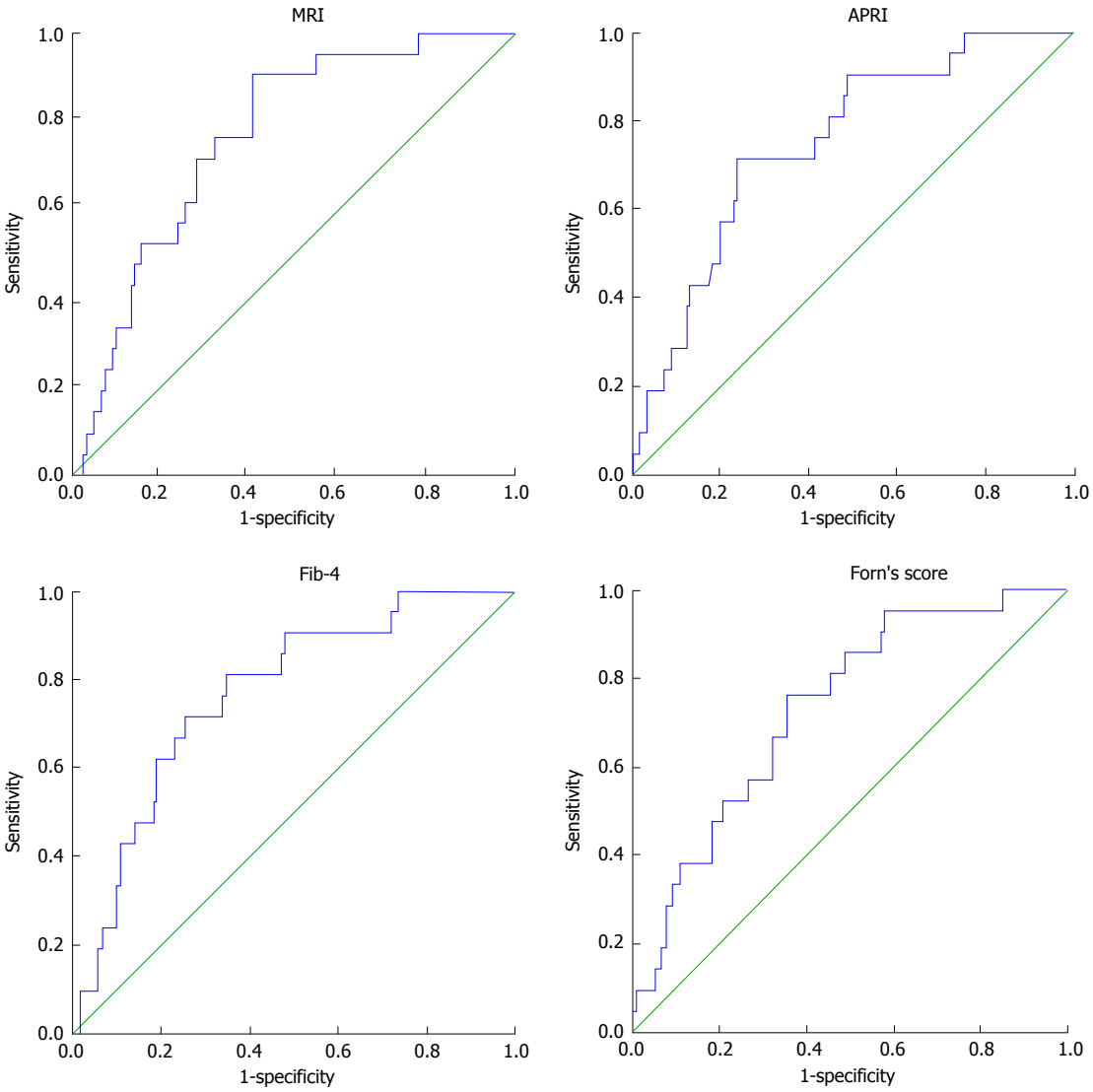

Figure 2 Receiver operating characteristic curve evaluating the cumulative incidence of liver-intervertebral disc ratio, aspartate amino transferase-to-platelet ratio index, Fib-4, and Forn's index. APRI: Aspartate amino transferase-to-platelet ratio index; MRI: Magnetic resonance imaging.

Table 2 Comparison of baseline characteristics between patients who have no hepatocellular carcinoma occurrence and hepatocellular carcinoma occurrence

Variables	No HCC occurrence n = 121	HCC occurrence n = 21	P value
Age (yr)	65.4 ± 12.9 (28-87)	70.3 ± 7.9 (51-79)	0.094
Sex (M/F)	59/62	11/10	0.759
AST (U/L)	46.0 ± 20.4 (11-110)	65.5 ± 31.6 (34-155)	0.000
ALT (U/L)	49.5 ± 32.5 (10-228)	64.4 ± 40.5 (31-205)	0.063
Serum albumin (g/dL)	4.1 ± 0.5 (2.4-5)	3.8 ± 0.5 (2.6-4.7)	0.030
Gamma-GT (IU/L)	67 ± 97 (14-811)	62 ± 43 (16-226)	0.829
ALP (U/L)	315 ± 158 (141-1206)	426 ± 212 (150-1006)	0.006
T.Chol (mg/dL)	178 ± 36 (90-280)	159 ± 35 (93-260)	0.029
T.Bil (mg/dL)	0.86 ± 0.42 (0.3-2.9)	0.86 ± 0.42 (0.3-2.9)	0.287
PT (%)	94 ± 15.5 (55.2-134)	86.8 ± 12.0 (67-110)	0.045
Platelet ($\times 10^3/\mu L$)	142 ± 61 (42-338)	104 ± 40 (46-166)	0.006
AFP (ng/mL)	11.7 ± 26.3 (1.6-235)	30.3 ± 31.6 (4.2-116)	0.004
LI	1.53 ± 0.20 (1.11-2.15)	1.37 ± 0.10 (1.23-167)	0.000
Patients who received IFN, n (%)	37 (30.6)	2 (9.5)	0.062
Patients who achieved SVR, n (%)	25 (20.7)	2 (9.5)	0.366

HCC: Hepatocellular carcinoma; AST: Aspartate amino transferase; ALT: Alanine aminotransferase; Gamma-GT: Gamma-glutamyl transpeptidase; ALP: Alkaline phosphatase; T.Chol: Total cholesterol; T.Bil: Total bilirubin; PT: Prothrombin time; AFP: α-fetoprotein; LI: Liver-intervertebral disc ratio; IFN: Interferon; SVR: Sustained virologic response; F: Female; M: Male.

Table 3 Risk factors contributing to hepatocellular carcinoma incidence

Variable	Univariate analysis			Multivariate analysis		
	Risk ratio	95%CI	*P*-Value	Risk ratio	95%CI	*P*-value
Age (per 1 year old)	1.04	1.01-1.09	0.045	1.05	0.99-1.11	0.139
Sex (F)	0.74	0.31-1.73	0.483			
AST (U/L)	1.02	1.01-1.03	< 0.001	1.01	0.99-1.03	0.200
ALT (U/L)	1.01	0.99-1.02	0.12			
Serum albumin (g/dL)	0.27	0.12-0.60	0.001	0.62	0.17-2.26	0.469
Gamma-GT (IU/L)	1.00	0.99-1.01	0.942			
ALP (U/L)	1.002	1.001-1.004	0.006	1.00	0.99-1.01	0.504
T.Chol (mg/dL)	0.98	0.97-0.99	0.01	0.99	0.98-1.01	0.483
T.Bil (mg/dL)	2.01	0.77-5.25	0.153			
PT (%)	0.97	0.94-0.99	0.018	1.01	0.97-1.05	0.621
Platelet ($\times 10^3/\mu L$)	0.98	0.97-0.99	0.003	0.99	0.98-1.01	0.281
AFP (≥ 10 ng/mL)	7.39	2.97-18.37	< 0.001	3.10	1.03-9.35	0.045
LI (< 1.46)	11.63	2.71-49.9	0.001	6.05	1.34-27.3	0.019
≥ 1.601	1.00					
1.501 to 1.60	2.68	0.17-42.9	0.48			
1.381 to 1.50	7.24	0.89-58.9	0.06			
1.311 to 1.38	11.5	1.2-103	0.02			
≤ 1.31	17.34	2.16-138.7	0.007			
Patients who received IFN	0.20	0.04-0.87	0.032	1.09	0.21-5.62	0.917
Patients who achieved SVR	0.35	0.81-1.51	0.158			

AST: Aspartate amino transferase; ALT: Alanine aminotransferase; Gamma-GT: Gamma-glutamyl transpeptidase; ALP: Alkaline phosphatase; T.Chol: Total cholesterol; T.Bil: Total bilirubin; PT: Prothrombin time; AFP: α-fetoprotein; LI: Liver-intervertebral disc ratio; IFN: Interferon; SVR: Sustained virologic response; F: Female.

Table 4 Analyses of liver-intervertebral disc ratio contributions to hepatocellular carcinoma occurrence risk divided by other risk factors

	Subgroup	*n*	Risk ratio	95%CI	*P*-value
Age	≥ 69	75	12.51	1.63-95.82	0.015
	< 69	67	9.2	1.11-76.58	0.041
Sex	Male	70	8.4	1.08-65.18	0.042
	Female	72	7.024	1.49-33.14	0.014
Platelet ($\times 10^3/\mu L$)	< 120	67	4.48	1.01-19.89	0.048
	≥ 120	75	14.96	1.89-118.2	0.013
Albumin (g/dL)	< 4.2	72	9.7	1.27-74.24	0.029
	≥ 4.2	70	10.79	1.29-89.7	0.028
ALT (U/L)	≥ 50	88	10.98	1.39-86.7	0.023
	< 50	54	12.7	1.62-99.63	0.016
IFN	-	103	13.35	1.78-100.1	0.011
	+	39	3.498	0.22-55.96	0.376
SVR	-	115	15.98	2.13-119.7	0.007
	+	27	3.795	0.28-60.74	0.346

ALT: Alanine aminotransferase; IFN: Interferon; SVR: Sustained virologic response.

0.627-0.840; APRI, 0.752 ± 0.05, 0.648-0.856; and Fib-4, 0.765 ± 0.05, 0.665-0.861. Comparing these results, MRI is as effective as the Fib-4 method and more effective than Forn's index and APRI.

Prognostic Factors of HCC occurrence risk by univariate and multivariate analyses

On univariate analysis, LI < 1.46, AFP ≥ 10, age (per year of age), AST (per 1 U/L), serum albumin (per 1 g/dL), ALP (per 1 U/L), T.Chol (per 1 mg/dL), PT (per 1%), platelets (per 1 × $10^3/\mu L$), and receiving IFN were identified as risk factors for the occurrence of HCC. The risk of HCC occurrence increased in accordance with LI decrease. On multivariate analysis, LI < 1.46 (P = 0.019) and AFP ≥ 10 ng/mL (P = 0.045) were identified as independent factors; LI: risk ratio: 6.05 (1.34-27.3, P = 0.019) and AFP: 3.1 (1.03-9.35, P = 0.045) (Table 3). The LI contributions to HCC occurrence risk were also evaluated in subgroup analyses. We investigated whether higher LI was a significant risk factor with several other factors (Table 4). High LI was a significant risk factor even with low or high values of age, Plt, albumin, ALT, and male or not, IFN-treated or not, and SVR achieved or not. The LI contribution was greater at age ≥ 69 (older group) and with platelets ≥ 120 × $10^3/\mu L$ (less fibrosis). In the IFN-untreated group and the SVR-unachieved group, there was a significant risk in low LI, but in the IFN-treated group and SVR not-achieved group, there

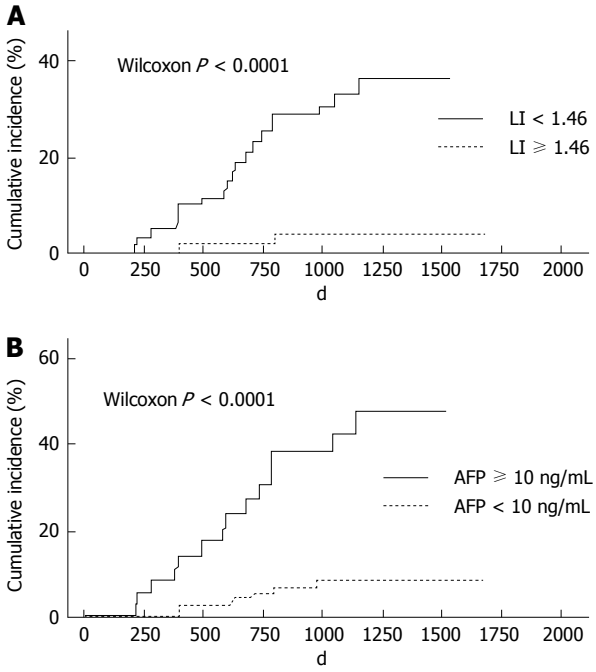

Figure 3 Relationship between cumulative occurrence rates and liver-intervertebral disc ratio (A), cumulative occurrence rates and serum α-fetoprotein level (B). A: Occurrence rates with LI < 1.46 were significantly higher than those with LI ⩾ 1.46; B: Occurrence rates with serum AFP ⩾ 10 ng/mL were significantly higher than in those with serum AFP < 10 ng/mL. LI: Liver-intervertebral disc ratio; AFP: α-fetoprotein.

were no significant differences because the sample numbers were very small.

Relationship between occurrence rate and LI or AFP

The occurrence rate in patients with LI < 1.46 was significantly higher than in those with LI ⩾ 1.46 (Wilcoxon $P < 0.0001$) (Figure 3A); in addition, in those with serum AFP ⩾ 10 ng/mL, it was significant higher than in those with serum AFP <10 ng/mL (Wilcoxon $P < 0.0001$) (Figure 3B).

DISCUSSION

It is known that liver fibrosis is the strongest prognostic factor of chronic liver disease and liver biopsy is now recognized as the best method for evaluating this condition[22], although it has problems such as complications. Several risk factors for HCC occurrence or recurrence have been reported, such as age, sex[1], serum albumin level[23,24], AFP level[25], and high transaminase[25]. Our study showed almost the same results as these previous reports. In particular, the progression of fibrosis may increase the risk of HCC incidence, so it is very important to determine the stage of liver damage[26,27]. Various methods have been reported for the evaluation of liver fibrosis and have been divided into two groups: ultrasonographic methods[15,17,28,29] and others[16,18]. Although gadolinium ethoxybenzyl diethylene triamine pentaacetic acid (Gd-EOB-DTPA)-enhanced MRI is one of the most sensitive methods to detect HCC development, it is also a very important method to evaluate liver fibrosis as a noninvasive investigation[19,30]. We used Gd-EOB-DTPA-enhanced MRI and the LI:EOB-MRI index = (post-liver intensity/post-disc intensity)/(pre-liver intensity/pre-disc intensity) because it has the highest accuracy of all of the calculation methods using EOB-MRI[18]. In this study, we used the 20-min hepatobiliary phase because many institutions accept the hepatobiliary phase as being 20 min after injection and it has also been accepted by consensus of the International Forum for Liver MRI[31]. In our previous study, the data between 20 and 25 min showed no significant difference (data not shown).

LI constantly decreased as the fibrosis stage progressed to a higher stage, but many values overlapped between close fibrous stages, so we decided that the best cut-off point was 1.46 on the ROC curve by calculating the accuracy value and likelihood ratio. Using this cut-off value, LI < 1.46 always showed a high risk, with both low and high risks for several other factors, showing that lower LI is a strong independent risk factor and can complement other risk factors. LI may reflect not only the fibrosis stage but also functional aspects of the liver because it is decided by various factors, such as decreased hepatocytes, deficient hepatocyte function, and indocyanine green clearance[32-34]. The uptake and excretion of gadoxetate disodium are carried out by the anion-transporting polypeptides Oatp1 and Mrp2[35]. The balance of these effects may regulate the signal intensity of liver parenchyma in the hepatobiliary phase followed by a decrease of its signal upon hepatic damage or deteriorating cirrhosis[36-38]. Viewed from this perspective, LI could be an outstanding predictor that reflects the occurrence of HCC and prognosis, in comparison to other methods that can assess only fibrosis.

In the present study, two patients developed HCC in the higher-LI group. According to their clinical data, both had significant splenomegaly and varices, and their actual pathology obtained from surgery was F4. OATP1B1/1B3 are hepatocyte-specific transporters determining the uptake of Gd-EOB-DTPA during MR, and genetic polymorphisms of their polypeptides might influence hepatic enhancement[39], but their actual influence is relatively small and the intensity in the second case was extremely high, so it was thought to be difficult to explain this discrepancy completely. In particular, one of the two patients achieved SVR during observation but developed HCC. AFP of the two patients did not change even when HCC developed. The occurrence of HCC after IFN therapy is a rare but important problem, as some studies have reported recently[40,41]. Chang *et al*[40] advocated calculating the HCC prediction score after IFN therapy and, using this method, the score of our case was 5 in the so-called medium-risk group. Because the AUROC value of Fib-4 is as high as that of LI, the cut-off value (4.0) of Fib-4 was determined because the highest accuracy (0.669) was obtained, with sensitivity: 80.9%, specificity:

64.5%, positive predictive value: 28.3%, and negative predictive value: 95.1%. Using this cut-off value, 4 patients developed HCC at < 4. Interestingly, although LI and Fib-4 have similar ROC, there are relatively weak correlations between these two methods (Pearson, r = -0.303, P = 0.0002), so it is thought that they complement each other. Our two cases in which HCC development initially could not be predicted were finally predicted using Fib-4. Therefore, a combination of these two methods is a better predictive method than using a single predictive method as they complement each other and, in addition to information on clinical advanced liver fibrosis, such as low Plt, splenomegaly, and the existence of obvious varices, they will enable more accurate prediction of HCC progression.

Methods such as LI using EOB-MRI and transient elastography may be strong predictors of the HCC occurrence risk. Fibroscan is more cost-effective than MRI, but the equipment is very expensive and is restricted for use in specific hospitals because it can be used only to evaluate tissue elasticity and is ineffective in patients who are obese or have ascites. On the other hand, MRI can evaluate patients who have ascites and/or are obese and is used in many general hospitals, so it is a widely available method.

Our study revealed that the EOB-MRI index is associated with the risk of HCC occurrence in hepatitis C patients and may become a substitute for liver biopsy when evaluating the risk in these patients, even when their condition is not appropriate for other noninvasive methods, and the combination of EOB-MRI index and Fib-4 may become a better predictive method of HCC progression.

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COMMENTS

Background

The major cause of cirrhosis is chronic hepatitis C and it is well known that the risk of occurrence of hepatocellular carcinoma increases as fibrosis progresses. Therefore, it is important to reveal the fibrosis stages of outpatients.

Research frontiers

The gold standard test to investigate the fibrous stage of the liver is needle biopsy, but it is potentially harmful, so other noninvasive methods are needed and several have been reported.

Innovations and breakthroughs

This method can predict the hepatocellular carcinoma (HCC) incidence noninvasively and has the advantage of being suitable for some individuals for whom other methods are unavailable due to several factors, such as the presence of ascites.

Applications

This method uses pictures that are typically obtained to detect HCC in outpatients, so it does not require the preparation of special equipment.

Peer review

The authors evaluated the development of HCC in hepatitis C virus patients using ethoxibenzyl-magnetic resonance imaging (EOM-MRI), and observed that EOM-MRI is a highly sensitive and predictive method. This study was well performed, and the manuscript is overall well written and easy to understand.

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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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